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Anti-Cytomegalovirus Activity of Atanyl Blue PRL, an Anthraquinone Derivative

Zohaib Alam

Virginia Commonwealth University

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ANTI-CYTOMEGALOVIRUS ACTIVITY OF ATANYL BLUE PRL, AN
ANTHRAQUINONE DERIVATIVE

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
at Virginia Commonwealth University.

by

ZOHAIB ALAM

Bachelor of Science, Bachelor of Arts, Virginia Commonwealth University, Richmond, VA,
2009

Major Director: Michael A. McVoy, Ph.D.
Professor, Department of Pediatrics and Microbiology and Immunology

Virginia Commonwealth University
Richmond, Virginia

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ABSTRACT

ANTI-CYTOMEGALOVIRUS ACTIVITY OF ATANYL BLUE PRL, AN ANTHRAQUINONE DERIVATIVE

By Zohaib Alam, MS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science
at Virginia Commonwealth University.

Virginia Commonwealth University, July 2013

Major Director: Michael McVoy, Ph.D.
Professor, Department of Pediatrics and Microbiology and Immunology

Cytomegalovirus (CMV) is a significant cause of mortality and morbidity in immunocompromised patients and an important cause of birth defects if acquired in utero. The licensed CMV antivirals, ganciclovir, cidofovir and foscarnet, all target the viral DNA polymerase. For each drug prolonged use is associated with significant toxicities and development of drug resistance. None are approved for use during pregnancy. Therefore, development of new anti-CMV drugs that target different pathways would be beneficial. All herpesviruses encode an alkaline nuclease. That genetic disruption of the CMV alkaline nuclease, UL98, reduces CMV replication by 1,000-fold suggests that UL98 may be a useful target for development of novel anti-CMV drugs. Moreover, using herpes simplex virus type 1 Hsiang and Ho found that the anthraquinone emodin inhibits activity of the viral alkaline nuclease, blocks viral replication in cell culture, and reduces viral pathogenesis in a mouse model (Brit. J. of Pharm., 2008). Earlier studies also showed that anthraquinone derivatives including emodin have anti-CMV activity (Barnard *et al.*, Antiviral Research 1992 & 1995), although the mechanism of CMV inhibition has not been further studied. We therefore sought to confirm the anti-CMV activities of emodin and related anthraquinone derivatives, to characterize

their mechanisms of action, and to determine specifically if they act through inhibition of UL98. Using a luciferase-based CMV yield reduction assay emodin inhibited CMV replication ($IC_{50} = 4.9 \mu M$); however, that the TD_{50} for cytotoxicity (determined using an luciferase-based cell viability assay) was only 2-fold higher suggested that emodin may act non-specifically. Two additional anthraquinone derivatives (acid blue 40 and alizarin violet R) inhibited CMV only at high concentrations ($IC_{50} = 238; 265 \mu M$) that were also cytotoxic. Atanyl blue PRL, however, exhibited anti-CMV activity ($IC_{50} = 6.3 \mu M$) with low cytotoxicity ($TD_{50} = 216 \mu M$). Thus, characterization of atanyl blue PRL (impact on gene expression, GFP expression, viral spread, infectivity, time of addition studies, and inhibition of UL98 nuclease activity) should be informative. Atanyl blue PRL appears to block immediate-early gene expression and reduce early and late gene expression. Atanyl blue PRL also blocked GFP expression, reduced viral spread, and also lowered the infectivity of CMV. Finally, atanyl blue PRL inhibits UL98 alkaline nuclease activity at an IC_{50} of $5.7 \mu M$. This suggests that atanyl blue PRL may inhibit CMV through inhibition of UL98. Thus, atanyl blue PRL represents a novel class of anti-herpesvirals and provides a lead structure for structure based drug discovery.

I. INTRODUCTION

History of CMV

Hippocrates documented an assortment of cutaneous lesions consistent with herpes simplex lesions. Greek scholars coined the term *herpes*, which means “to creep or crawl”, in reference to the spreading nature of herpetic lesions. Herpes viruses infect virtually all vertebrates and invertebrates and are one of the most common viruses found in humans (35). Cytomegalovirus, or CMV, belongs to the *Herpesviridae* family of viruses (1). The *Herpesviridae* family of viruses is a large family of double stranded DNA (120 to 235 kb) viruses. The DNA is encased in an icosahedral capsid and an outer protein matrix layer known as tegument (9). The *Herpesviridae* family is divided into three subfamilies: alphaherpesvirinae, betaherpesvirine, and gammaherpesvirinae. The alphaherpesvirinae are described as having rapid growth and spread in cell cultures. They infect fibroblasts and epithelial cells to replicate lytically and establish latency in sensory neurons. Examples of the alphaherpesvirinae are herpes simplex virus type 1 and type 2 (HSV-1, HSV-2) and varicella zoster virus. The betaherpesvirinae are characterized by slow infection and growth in cell culture and a long replication cycle. They can infect various cell types in lytic infection and can establish latency in lymphoid progenitor cells. Examples of the betaherpesvirinae are CMV, human herpesvirus 6, and human herpesvirus 7. The gammaherpesvirinae are known to have limited growth in lymphoblastoid cells and to establish latency in B or T cells. Examples of the gammaherpesvirinae are Epstein-Barr virus and Kaposi’s sarcoma-associated herpesvirus (KSHV) (35).

Molecular Biology of CMV

CMV has a genome of 235 kb, the largest of any herpesvirus, encoding over 200 genes within a 100 nm diameter icosahedral capsid (10). The capsid is surrounded by a proteinaceous tegument, which is further surrounded by a glycoprotein and lipid bilayer envelope. The envelope is on the surface of the virus and contains glycoproteins such as gB, gN, gO, gH, gM, and gL. These glycoproteins function in virus-cell binding, cell fusion, virus entry into host cells, cell-to-cell spread, and virion maturation. Mutations in these glycoproteins can produce non-infectious progeny virus (11).

The CMV genome is expressed temporally in a regulated cascade leading to synthesis of three categories of viral proteins: Immediate-early, early, and late (figure 1). Immediate-early genes are the first viral genes to be expressed after a cell is infected and do not require the expression of other viral genes or proteins. Immediate-early gene products regulate the expression of early and late genes and the expression of early genes is dependent on the presence of immediate-early proteins. Early genes encode non-structural proteins such as viral DNA replication factors needed for DNA synthesis, DNA repair enzymes, and proteins involved in immune evasion. After the early proteins are synthesized viral DNA replication can occur. Late genes are only expressed after viral DNA synthesis begins. Late proteins are mainly involved in structural roles and the assembly and morphogenesis of the virion. The early and late proteins function together to allow the newly synthesized genomes to mature and package (11).

CMV Life Cycle

Virus attachment to the cell surface involves low affinity interaction of gB to heparin sulfate proteoglycans followed by a stronger binding of gB with its nonheparin receptor. The

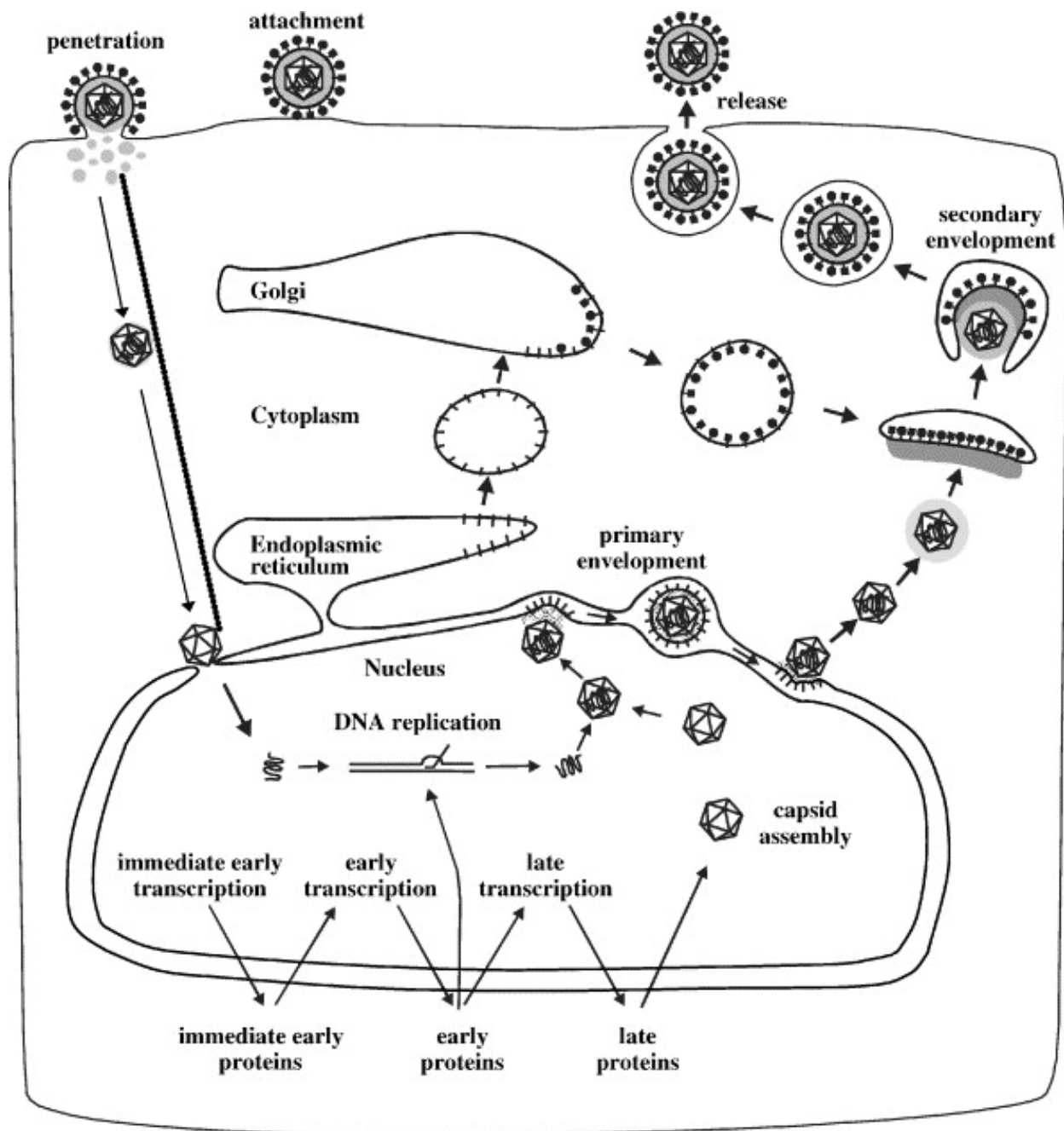


Figure 1: Life Cycle of Herpesviruses: The virus enters the cell by attachment and penetration or endocytosis. Viral capsid and tegument proteins are released into the cytoplasm and the capsid is transported to the nucleus via microtubules. The viral capsid docks with the nucleopore and the viral dsDNA is released into the host nucleus. Immediate-early, early, and late gene expression and cytoplasmic translation of viral mRNA occur. Capsid proteins are transported to the nucleus from the cytoplasm and empty capsids are assembled. Viral DNA is packaged into the capsid and the capsid exits the nucleus, gains tegument, and gains its envelope as it exits the cell (21). Reprinted from Virus Research, 106/2, Mettenleiter TC, Budding events in herpesvirus morphogenesis, 167-180, Copyright (2004), with permission from Elsevier.

final interaction involves the fusion of the viral envelope with the cell membrane, allowing viral penetration (11). In some cell types such as endothelial and epithelial cells, viral entry occurs via endocytosis (36). After viral penetration, the capsid and tegument proteins are released into the cytoplasm and are translocated to the nucleus via microtubules (figure 1). The viral capsid docks with the nuclear pore and the viral dsDNA is released into the nucleus (37). Immediate-early, early, and late gene expression and cytoplasmic translation of viral mRNA occurs. Viral DNA circularizes in the nucleus. Replication of the circular viral DNA forms concatemeric DNA in the nucleus. Capsid proteins are transported to the nucleus and empty capsids are assembled. During the late stages of viral DNA replication concatemeric DNA is packaged into capsids and cleaved to unit length. The capsids acquire a primary envelope by budding at the inner nuclear membrane and followed by a de-envelopment at the outer nuclear membrane. The capsid now enters the cytoplasm and further matures by gaining tegument and the final glycoprotein-containing envelope as it buds into a vesicle (38). The vesicle is transported to the cell surface where it fuses with the cell membrane and mature progeny virus is released to the extracellular environment where it can infect more cells (figure 1) (13).

Significance and Impact of CMV on Human Health

CMV is a relatively common infection with seroprevalence of 60% or more in persons greater than 50 years of age. It is estimated that between 45 to 100% of women of reproductive age are seropositive for CMV. The portion of women who are seronegative for CMV are at risk of primary CMV infection during pregnancy and bear a risk of giving birth to babies who will suffer from CMV-related disabilities. Seroprevalence tends to be high in South America, Africa,

and Asia (>90%), and seroprevalence is also higher with non-whites and with individuals of lower socioeconomic status. Seroprevalence is almost 60% in United States and Europe. (2)

Most people infected with CMV are asymptomatic; however after primary infection some may show mild fever or mononucleosis-like symptoms and the host will remain infected with CMV in the latent phase for life. They may shed virus in urine, saliva, semen, cervical secretions, and breast milk and contact with these secretions can lead to transmission of CMV. For immunocompromised and immunosuppressed patients, CMV is a significant cause of mortality and morbidity. CMV is also an important cause of birth defects in newborns if acquired in utero. (39)

CMV Infection and Disease Among Transplant Recipients

CMV is a major cause of mortality and morbidity in patients undergoing stem cell and solid organ transplantation (40). Due to the opportunistic behavior under immunosuppression, CMV infection has a large impact on transplant recipients. Active CMV infection occurs in 30-75% of transplant recipients and has a mortality rate of approximately 5%. The type of organ transplant has a role in acquiring CMV infection. Liver, lung, and pancreas transplant recipients have a high risk of acquiring CMV infection, whereas recipients of heart, small bowel, and kidney transplants have a lower risk (41). Disease results from viral invasion of organ systems or effects on the immune system that can increase the risk of other infections or promote acute graft rejection (40). Clinical manifestations of CMV are CMV pneumonia, gastrointestinal disease, hepatitis, CNS disease, retinitis, nephritis, cystitis, myocarditis, pancreatitis, CMV syndrome, and CMV-associated graft failure (3). Several diseases are linked to CMV infection with transplantation such as transplant rejection, transplant dysfunction, accelerated coronary

atherosclerosis after heart transplantation, vanishing bile duct syndrome following liver transplantation, constrictive bronchiolitis syndrome subsequent to lung transplantation, and the development of post-transplant lymphoproliferative disease (4). Severe disease is most prevalent when a seronegative recipient receives organs or blood products from a seropositive donor; this can lead to primary infection. However, for stem cell transplant recipients, the risks are reversed, severe disease is most prevalent when a seropositive recipient receives a stem cell transplant from a seronegative donor (51). CMV disease can also occur following reactivation of the virus from a latent state in seropositive transplant recipients. Furthermore, if the donor and recipient are both seropositive but with two distinct strains of CMV, then it can lead to a superinfection or reinfection of CMV (41).

CMV in HIV-Infected Patients

CMV is the most frequent opportunistic pathogen in patients infected with HIV. Upwards of 45% of patients with AIDS are afflicted with CMV disease at some point during the course of their AIDS disease. CMV can affect the eyes, gastrointestinal tract, lungs, liver, and central nervous system, and can cause diseases such as retinitis, colitis, pneumonitis, hepatitis, and radiculopathy. CMV retinitis accounts for 85% of all CMV disease in patients with AIDS and untreated retinitis spreads through the retina and causes retinal destruction and blindness (5). Despite current therapies, most patients with CMV retinitis undergo relapse.

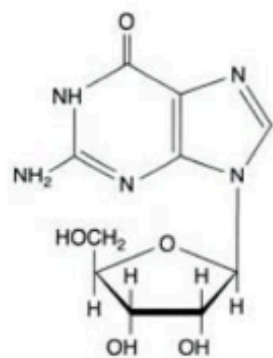
Congenital and Perinatal CMV

In the United States, 40,000 children or 1% of all live births are born with an active CMV congenital infection. However, only 8,000 or 20% of those births are symptomatic. CMV

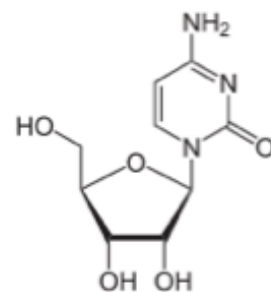
infection is the most common congenital infection and a major cause of morbidity and mortality among infants (7). Infection can occur in utero or perinatally by transmission from mothers who are infected with primary CMV infection or have CMV reactivation. It can also occur after birth due to contact with secretions such as breast milk or saliva of a seropositive mother (18). In the United States, approximately 50% of women of childbearing age are seronegative and susceptible to primary CMV infection and up to 8% of seronegative mothers will become infected with CMV during pregnancy. Of those mothers who are infected during pregnancy, 50% will transmit CMV to their fetus, and approximately 28% of those fetuses will have symptoms at birth (8). However, if the mother is seropositive for CMV prior to pregnancy then the rate of infection of the fetus is 0.15 to 1.5%, displaying that prior maternal infection confers substantial protection against congenital infection and reduces the severity of disease (42). Symptomatic children may have mental retardation, cerebral palsy, sensorineural hearing loss, impaired vision, microcephaly, encephalopathy, seizures, chorioretinitis, and hepatosplenomegaly (43). Hearing loss occurs in approximately 40% of symptomatic infants with congenital CMV infection (43). Fifteen-percent of infants with asymptomatic congenital CMV infection at birth will later develop hearing loss (44).

CMV Antivirals

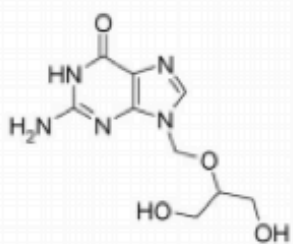
Currently there are several drugs approved for the treatment of CMV infections: Ganciclovir and its prodrug Valganciclovir, Foscarnet, Cidofovir, Acyclovir and its prodrug Valacyclovir, and Fomivirsen (shown in figure 2). Ganciclovir and its prodrug Valganciclovir, Foscarnet, Cidofovir are approved for systemic CMV treatment. Valacyclovir is approved for prophylaxis of renal transplant recipients and other solid organ transplants (outside



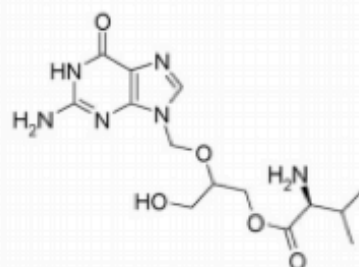
Guanine



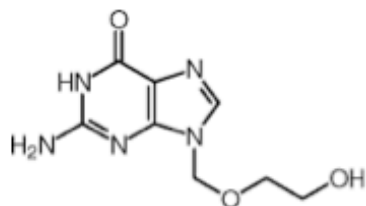
Cytidine



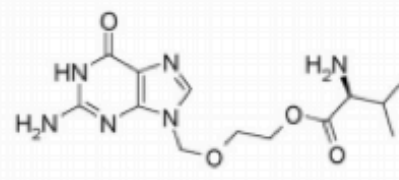
Ganciclovir



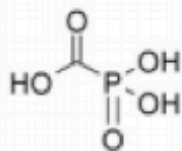
Valganciclovir



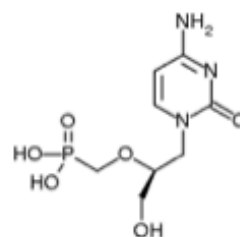
Acyclovir



Valacyclovir



Foscarnet



Cidofovir

5'-GCGTTTGCTCTTCTTGCG-3'

Fomivirsen

Figure 2: Current antivirals used for treating CMV infections: Guanine and cytosine nucleosides are shown on top with ganciclovir, valganciclovir, acyclovir, valacyclovir, foscarnet and cidofovir also shown.

the United States). Fomivirsen is approved for local treatment of CMV retinitis. Unfortunately these therapies are inadequate. They can show significant toxicities and limited efficacy. Furthermore, due to their potential toxicities, none are approved for congenital use. They can also be limited by a low percentage of the drug reaching systemic circulation and a risk of drug resistance with prolonged use (13). With the exception of fomivirsen, all approved drugs target the viral DNA polymerase, so there is likelihood that mutations in the DNA polymerase may render these drugs ineffective (13).

Ganciclovir and Valganciclovir

Ganciclovir, also known as DHPG (9-(1,3-dihydroxy-2-propoxymethyl)guanine), was the first antiviral approved for use against CMV and continues to be the drug-of-choice for CMV infection and CMV disease in transplant recipients. Approved in 1989, Ganciclovir is a nucleoside analog of 2-deoxyguanosine and is marketed under the names Cytovene, Cymevene, and Vitrasert. Ganciclovir is first phosphorylated by the viral kinase UL97 to its monophosphate form (figure 3). After the initial phosphorylation by the viral kinase it is further phosphorylated to its diphosphate form then triphosphate form by host cell kinases (figure 3). The triphosphate form of ganciclovir competes with deoxyguanosine triphosphate (dGTP) incorporation during new viral DNA synthesis by the viral DNA polymerase (UL54) (figure 3). The incorporation of ganciclovir inhibits the addition of dGTP into the viral DNA and the synthesis of the viral DNA is markedly diminished (13, 45). Mutations in UL97 or UL54 can lead to resistance to ganciclovir (14). UL97 mutations generally emerge first and confer a lower level of resistance in comparison to UL54 mutations, which emerge later and have a higher level of drug resistance (16). Ganciclovir has a low oral bioavailability (~5-6%)

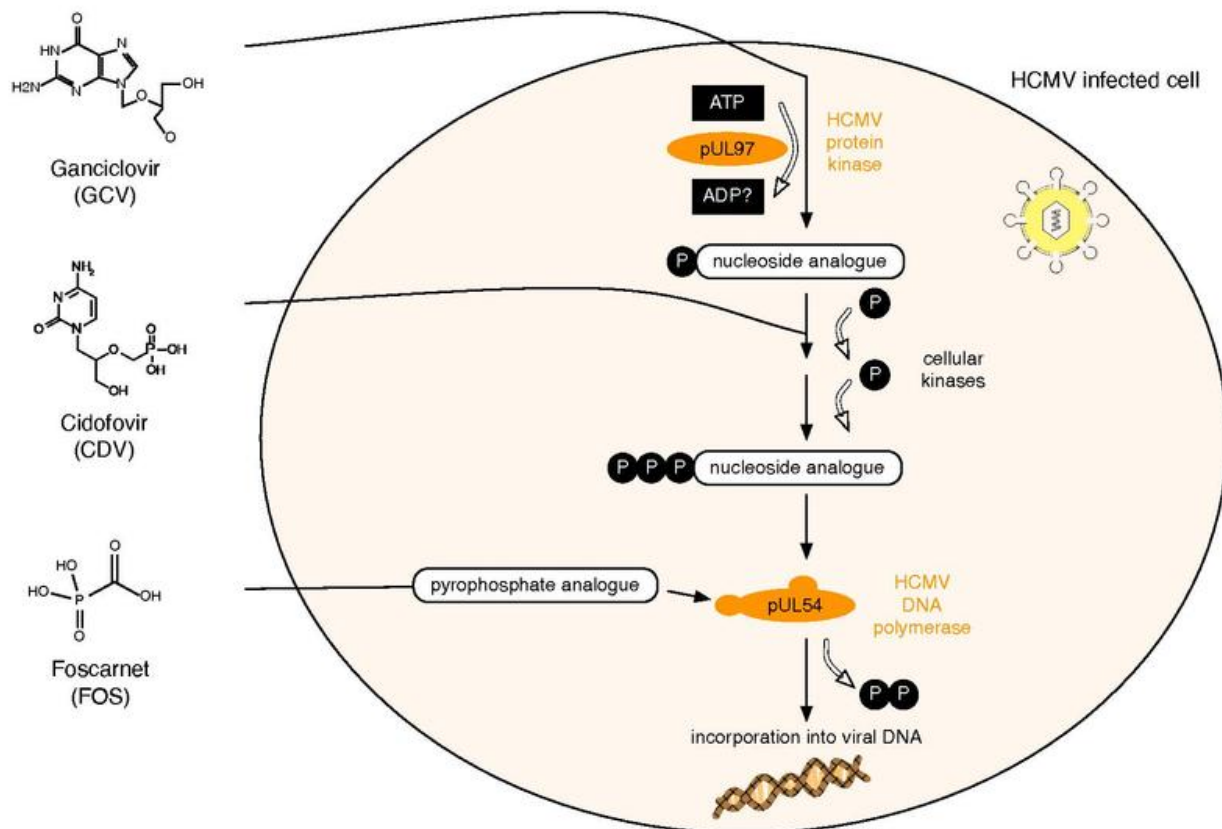


Figure 3: Mechanism of action of ganciclovir, cidofovir, and foscarnet: Ganciclovir is a nucleoside analog of 2-deoxyguanosine. Ganciclovir is first phosphorylated by the viral kinase UL97 to its monophosphate form. After the initial phosphorylation by the viral kinase it is further phosphorylated to its diphosphate form then triphosphate form by host cell kinases. The triphosphate form of ganciclovir competes with dGTP incorporation during new viral DNA synthesis by the viral DNA polymerase (UL54). The incorporation of ganciclovir triphosphate inhibits the addition of dGTP into the viral DNA. Cidofovir is an acyclic nucleoside phosphonate. This nucleoside analog inhibits chain elongation by inhibiting the incorporation of dCTP into the nascent viral DNA. Cidofovir is a monophosphate that is converted to the active diphosphate form by host cell kinases. Cidofovir diphosphate acts as a competitive inhibitor to dCTP and incorporates into the viral DNA blocking the function of the viral DNA polymerase leading to premature chain termination of viral DNA synthesis. Foscarnet inhibits the function of the viral DNA polymerase by binding to the pyrophosphate binding site and inhibiting the release of pyrophosphate from the terminal nucleoside triphosphate on the growing DNA chain.

however its prodrug, valganciclovir, has a much higher oral bioavailability (~60%). Valganciclovir is a L-valyl ester of ganciclovir and is converted to ganciclovir by intestinal and hepatic esterases (15).

Ganciclovir is used to treat CMV retinitis in AIDS patients, prevention of CMV disease in solid organ and heart and bone marrow transplant patients, and prevention of CMV infection in advanced HIV patients who are at risk for CMV disease (13).

Ganciclovir is associated with hematologic side effects such as neutropenia, anemia, and thrombocytopenia. It is also associated with reproductive toxicity and aspermatogenesis. Ganciclovir is also classified as a potential human carcinogen, teratogen, and mutagen. These toxicities and the potential for drug resistance to ganciclovir limit the therapeutic usefulness of ganciclovir. Furthermore, ganciclovir is not approved for the treatment of congenital CMV infections (13).

Foscarnet

Foscarnet, also known as phosphonoformic acid, was the second drug approved for use against CMV infections. Foscarnet is generally considered a second-line therapy for the treatment of CMV infections, however, it is the preferred drug for patients who have ganciclovir resistance or have dose-limiting neutropenia. Approved in 1991, foscarnet is a pyrophosphate analog and is marketed under the name Foscavir. Foscarnet inhibits the function of the viral DNA polymerase by binding to the pyrophosphate binding site and inhibiting the release of pyrophosphate from the terminal nucleoside triphosphate on the growing DNA chain (figure 3). Unlike ganciclovir, foscarnet does not require host or viral kinases to function, however, similar to ganciclovir, resistance mutations map to *UL54*. Further, cross-resistance has also been seen

between ganciclovir and Foscarnet. Foscarnet is mainly used for treatment of CMV retinitis in patients with HIV infection. However, foscarnet is associated with nephrotoxicity and electrolyte imbalance that may cause cardiac or neurological disorders and even death. (13). Foscarnet has an oral bioavailability of 12-22% and is not approved for use with congenital CMV infections (13, 17).

Cidofovir

Cidofovir, also known as 1-[(S)-3-hydroxy-2-(phosphonomethoxy)propyl] cytosine dihydrate, was the third drug approved for use against CMV disease. Cidofovir is a broad-spectrum antiviral drug that can be used against other herpesviruses and other DNA viruses such as the smallpox virus. Approved for use against CMV in 1996, cidofovir is an acyclic nucleoside phosphonate and is marketed under the name Vistide. Cidofovir is a monophosphate that is converted to the active diphosphate form by host cell kinases. Cidofovir diphosphate acts as a competitive inhibitor to dCTP and incorporates into the viral DNA, blocking the function of the viral DNA polymerase leading to premature chain termination of viral DNA synthesis. Unlike ganciclovir, cidofovir does not need the viral kinase UL97 for the first phosphorylation. However, like ganciclovir, mutations in the viral DNA polymerase gene *UL54* can lead to resistance to cidofovir. Cidofovir is mainly used for the treatment of CMV retinitis in AIDS patients and can also be used in patients who have developed a resistance to ganciclovir due to *UL97* mutations. It can also be used with transplant patients. However, cidofovir is associated with significant nephrotoxicity and neutropenia and is also classified as a potential human carcinogen and teratogen (13). Cidofovir has an oral bioavailability of less than 5% and is not approved for use with congenital CMV infections (13, 17).

Acyclovir and Valacyclovir

Acyclovir, also known as acycloguanosine or acyclovir, is another antiviral drug that can be used against CMV infections. Like ganciclovir, acyclovir is an analog of 2'-deoxyguanosine and is marketed under the names Cyclovir, Herpex, Acivir, Acivirax, Zovirax, Zoral, and Xovir. Also like ganciclovir, acyclovir is first phosphorylated by the viral kinase UL97 to its monophosphate form. After the initial phosphorylation by the viral kinase it is further phosphorylated to its diphosphate form then its active triphosphate form by the host cell kinases. The triphosphate form of acyclovir leads to chain termination during new viral DNA synthesis by UL54. The incorporation of acyclovir inhibits the addition of dGTP into the viral DNA and the synthesis of the viral DNA is inhibited. Further, like ganciclovir, mutations in *UL97* or *UL54* can lead to resistance to acyclovir. However, unlike ganciclovir, acyclovir is a less efficient substrate for host cell kinases leading to less potency, lower intracellular concentrations, and lower toxicity. Acyclovir has a low bioavailability (~6-10%), however its prodrug, valacyclovir has a considerably higher oral bioavailability (55%). (13). Valacyclovir is the L-valyl ester of acyclovir and is converted to acyclovir by hepatic esterases (15).

Acyclovir is not approved for use with CMV disease, however it is a very potent drug against some viruses. It gained approval in 1982 and is commonly used in the treatment of HSV and varicella zoster infections. It is also used with transplant patients to prevent the HSV reactivation and can also be used with neonatal HSV infections (13). Acyclovir has been safely used in pregnant women to reduce active genital lesions and transmission to newborns (13).

Even though acyclovir has limited efficacy against some herpesviruses such as Epstein-Barr virus and CMV, the use of acyclovir against CMV disease has been studied (19). When acyclovir was used in solid organ transplant patients, a 56% reduction in CMV infection and a

59% reduction in CMV disease was seen in comparison to patients receiving placebo or no therapy. Similar results were seen with valacyclovir. In Europe, Valacyclovir is approved for use against CMV infection and disease in renal, heart, and solid organ transplant patients (13).

Fomivirsen

Fomivirsen is another antiviral drug that is used against CMV infection. It is the first anti-sense antiviral to be approved by the FDA (20). Fomivirsen is a 21-nucleotide anti-sense RNA with the sequence 5'-GCG-TTT-GCT-CTT-CTT-GCG-3'. Approved in 1998, it is marketed under the name Vitravene. Fomivirsen is complementary to the mRNA sequence of CMV immediate-early-2 gene (IE2); hence it specifically targets the immediate-early transcriptional unit of CMV. Fomivirsen is mainly used as a second-line therapy for treatment of CMV retinitis in AIDS patients who are resistant to ganciclovir or have dose-limiting adverse reactions to ganciclovir. Fomivirsen is not used systemically; rather it is used locally by the administration of an intraocular injection one to two times every four weeks. The most common adverse effect is an ocular inflammation known as uveitis, however it can be treated by topical corticosteroids (13).

UL98

Alkaline nucleases are highly conserved throughout the Herpesvirus family (50). The protein UL98 is an alkaline nuclease encoded by the *UL98* gene of CMV and has both endonuclease and exonuclease activity; however the function of alkaline nucleases are not fully understood (50). Alkaline nucleases have optimal nuclease activity at alkaline pH environments (50). Alkaline nucleases may also be involved in viral DNA processing and capsid egress from

the nucleus (29). It has been previously described that emodin specifically inhibits the activity of HSV-1 alkaline nuclease, UL12. Emodin treatment of HSV infected cells results in accumulation of capsids in the nucleus (22). Further, it has been shown that CMV *UL98* null mutants are viable. However, viral replication was reduced by up to 1,000-fold, indicting *UL98* is an important though not essential CMV gene (50). Due to the need for CMV antivirals with alternative targets of action and lower toxicity, investigation of UL98 could lead to a novel target for the development of new CMV antivirals.

Objectives

Current therapy against cytomegalovirus is sub-optimal. Antiviral drugs have incomplete effectiveness against CMV infections and substantial dose-limiting toxicities with prolonged use. None are approved to for use with congenital infections, and patients develop resistance to the drugs. Further, the most commonly used drugs act by inhibiting the viral DNA polymerase, so there is a chance of cross-resistance of drugs. Countless children develop permanent disabilities and many children and adults die each year because there are no other options for the treatment of their CMV disease.

Therefore, we seek to study new drugs and drug targets that can be used against CMV. New drugs would give more options for those suffering with CMV disease. A new drug target could open up an entire new area of antiviral research and development of a new class of CMV drugs, especially for patients resistant to current therapy. New drugs could also be used synergistically with current therapies to improve effectiveness. Novel drugs might also have lower toxicity and be safe to use with congenital infections. It has been previously reported that anthraquinone derivatives have antiviral activity against herpes simplex and CMV, however the

mechanism of this action is uncertain (23, 24). We have evaluated anthraquinone derivatives emodin, atanyl blue PRL (also known as acid blue 129), acid blue 40, and alizarin violet R in this study.

Anthraquinones

Anthraquinone, also known as anthracenedione or dioxoanthracene, is an aromatic organic compound with the chemical formula $C_{14}H_8O_2$ (figure 4). Anthraquinone derivatives have this general structure, but may have various substituents on C-1, C-2, C-3, C-4, C-5, C-6, C-7, and C-8. Synthetic dyes are often derived from anthraquinones (25). Anthraquinones can be used for the industrial production of hydrogen peroxide and also as a laxative (26, 27). Anthraquinones are present in many plants extracts such as *Rheum officinale*, *Aloe barbadensis*, *Rhamnus frangula*, *Rhamnus purshianus*, *Cassia angustifolia*, *Hypericum perforatum*, and *Polygonum genera* (22, 28). As discussed further below, anthraquinones have been shown to be directly virucidal to certain enveloped and RNA and DNA viruses (28).

Antiviral activity of Anthraquinone Derivatives

The antiviral activity of anthraquinone derivatives has been investigated. In 1951, it was reported that hypericin, an anthraquinone dimer isolated from the plant *Hypericum perforatum*, has both antiviral and virucidal activity (32). Synthetic derivatives of anthraquinone can also have *in vivo* antiviral activity against picornavirus encephalomyocarditis (EMC) virus in mice (33). In 1979, it was reported that a hypericin-containing topical ointment helped in the healing of herpes simplex infections (32). It is also reported that hypericin inhibited the replication of influenza virus A and B, both *in vitro* and *in vivo*, and herpes simplex virus *in vitro* (32).

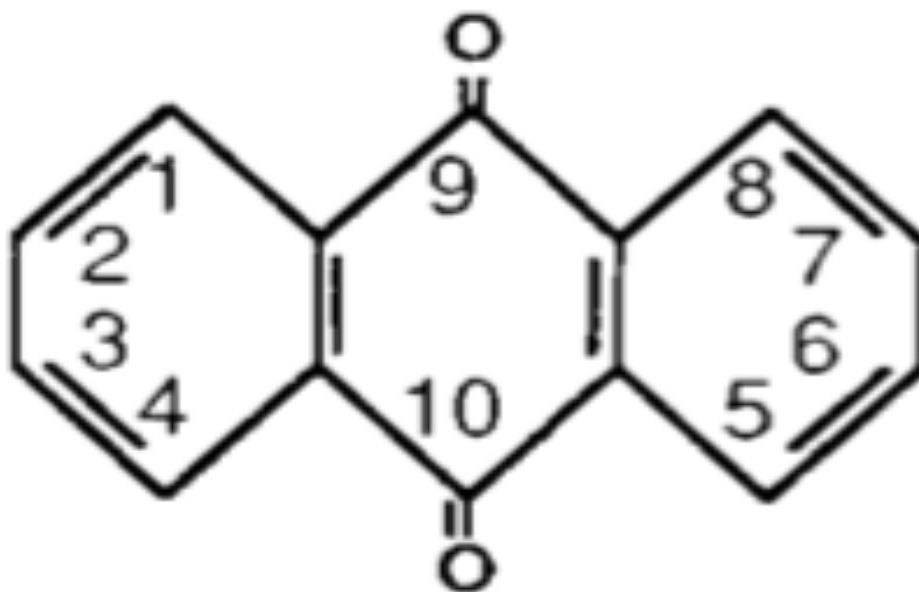


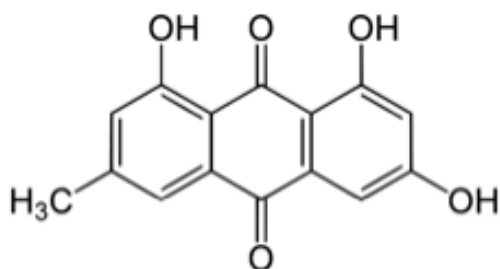
Figure 4: Chemical structure of Anthraquinone (9,10-dioxoanthracene): Anthraquinone derivatives have this general structure, but may have various substituents on C-1, C-2, C-3, C-4, C-5, C-6, C-7, and C-8. The numbering system is not consistent with Chemical Abstracts or with IUPAC nomenclature; it is shown this way for ease of comparison with derivatives. (24)

In addition, it has been reported that hypericin has activity against murine retroviruses and Moloney murine leukemia virus (32). Anthraquinones and their derivatives have demonstrated activity against human immunodeficiency virus (HIV) and other retroviruses, herpes simplex virus type 1 and type 2, influenza virus, vesicular stomatitis virus, Epstein-Barr virus, Murine Friend leukemia virus, and poliovirus (23, 24, 32, 33). It has also been shown that aloe emodin, an anthraquinone prepared from *Aloe vera*, is able to inhibit HSV-1 and HSV-2 *in vitro* and *in vivo* (33, 34). Relevant to our interest in CMV UL98 as a potential antiviral target, emodin was also shown to inhibit enzymatic activity of the HSV-1, alkaline nuclease, UL12, *in vitro*. Moreover, in 1992 anthraquinone derivatives were studied for antiviral activity against CMV (24). Several anthraquinone derivatives showed activity against CMV, including emodin, atanyl Blue PRL, acid blue 40, and alizarin violet R (23, 24). Their half-maximal inhibitory concentrations (IC_{50}) were 4.1 μ M, 7 μ M, 10 μ M, and 10 μ M respectively (23, 24).

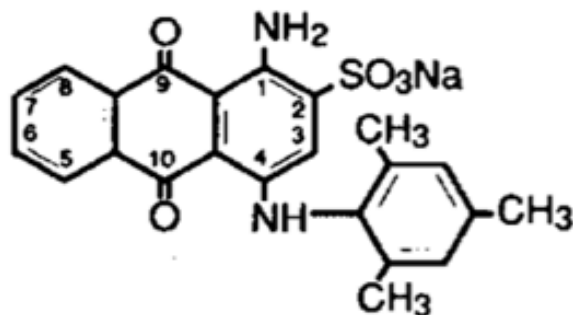
Summary

Since most of the currently approved antiviral therapies against CMV target the viral DNA polymerase, there is a high chance of drug resistance with prolonged use. Additionally, these drugs are associated with significant dose-limiting toxicities such as neutropenia and nephrotoxicity. Therefore, there is a substantial need for alternative anti-CMV compounds with novel, non-nucleoside targets of inhibition which are also less toxic to the patients. Having non-nucleoside drugs with a different mechanism of action than the current antivirals may allow the treatment of congenital infections, give patients more options for treatment, and most importantly, it could save the lives of countless adults and children. Understanding and

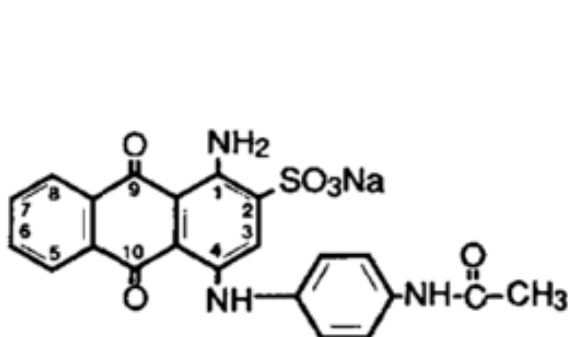
identifying the mechanism of action of anthraquinone derivatives may allow us to identify a novel target of inhibition against CMV.



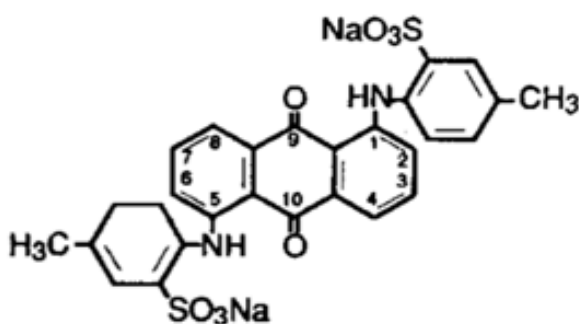
Emodin



Atanyl Blue PRL (Acid Blue 129)



Acid Blue 40



Alizarin Violet R

Figure 5: Chemical Structure of Anthraquinone derivatives used in this study: Emodin, atanyl blue PRL (also known as acid blue 129), acid blue 40, and alizarin violet R are shown. The numbering system is not consistent with Chemical Abstracts or with IUPAC nomenclature; it is shown this way for ease of comparison with derivatives. (23).

II. MATERIALS AND METHODS

Cells

Human fetal lung fibroblast (MRC-5) cells (ATCC CCL-171) were obtained from American Type Culture Collection (ATCC). MRC-5 cells were grown in minimum essential medium (Gibco-BRL) supplemented with 10% fetal calf serum (HyClone Laboratories), 10,000 IU/ml penicillin, 10 mg/ml streptomycin, and 29.2mg/ml L-glutamine (Gibco-BRL) (MEM). All cell cultures were maintained at 37°C in a 5% carbon dioxide (CO₂) atmosphere.

Viruses

CMV strain BADrUL131-Y4 was a gift from Dai Wang and Thomas Shank. It was derived from a BAC clone of the CMV strain AD169 genome that had been modified in *E.coli* by Wang and Shenk to contain a green fluorescent protein reporter cassette for efficient detection and quantification of viral infection (46). Subsequently the *UL131* mutation was repaired to express a functional UL131 protein that allows efficient entry and replication in both MRC-5 cells and ARPE-19 epithelial cells (47). Strain RC2626 is a Towne strain CMV. A luciferase expression cassette was introduced into the *US2-US6* region to create recombinant virus RC2626 (48). Due to mutation in *UL130*, RC2626 can replicate efficiently in fibroblasts but not in epithelial cells.

Drugs

Ganciclovir was purchased from InvivoGen. Fifteen potential UL98 inhibitors were acquired from the Synthesis and Chemistry Branch, Developmental Therapeutics Program, Division of Cancer Treatment and Diagnosis, National Cancer Institute. Emodin, atanyl blue

PRL and acid blue 40 were purchased from Sigma-Aldrich Co., and alizarin violet R was purchased from MP Biomedicals. To produce stock solutions, ganciclovir was solubilized in water at a concentration of 100 mM. Emodin, atanyl blue PRL, acid blue 40, and alizarin violet R were dissolved in dimethyl sulfoxide (Sigma-Aldrich company) at a stock concentration of 100 mM. The potential UL98 inhibitors were dissolved in dimethyl sulfoxide at a stock concentration of 10 mM. The compounds were stored at -20 degrees Celsius.

Luciferase Reporter Gene Assay for Anti-Cytomegalovirus Activity (Protocol based on an assay developed by Bhawe *et al.* (2013), Antiviral Research in press)

Clear-walled, clear-bottom 96-well plates containing confluent monolayers of MRC-5 cells were infected with RC2626 virus at a multiplicity of infection (MOI, ratio of infectious virus to cell) of 0.03 and incubated for 1 hour to allow viral entry. Eleven two-fold serial dilutions of emodin and six three-fold dilutions of atanyl blue PRL, acid blue 40, or alizarin violet R were prepared in 200 μ l MEM in triplicate in a 96-well format. These compound dilutions were transferred to infected cells to produce final drug concentrations of 0.24 to 250 μ M for emodin and 1.03 to 250 μ M for atanyl blue PRL, acid blue 40, and alizarin violet R. UL98 inhibitor compounds were also prepared in 200 μ l MEM in triplicate in a 96-well format and used at 25 μ M and 100 μ M final concentrations. Triplicates of no drug, no virus, and ganciclovir controls were included on each plate.

After incubation for 5 days, 50 μ l supernatants from the 96-well plate above were transferred to a black-walled, clear-bottom 96-well plate containing confluent MRC-5 monolayers. After 24 hours, 100 μ l Steady-Glo luciferase assay reagent (Promega) was added. After allowing 10 minutes for the luciferase assay reagent to lyse the cells, the luciferase activity

was measured in relative light units (RLU) using Biotek Synergy HT Multimode Microplate Reader. To allow easier comparability between experiments, the data were normalized by converting RLU to “percent maximum luminescence” for each experiment. The data were fitted with a nonlinear variable slope four-parameter regression curve and the half-maximal inhibitory concentrations (IC_{50}) (drug concentrations at which luciferase signal was reduced by half) of the test compounds were determined using Prism software by GraphPad, Inc.

Luciferase-Based Toxicity Assay

Black-walled, clear bottom 96-well plates containing confluent monolayers of MRC-5 cells were incubated with eleven two-fold serial dilutions of emodin or six three-fold dilutions of atanyl blue PRL, acid blue 40, or alizarin violet R were prepared in 200 μ l MEM in triplicate in a 96-well format. These compound dilutions were transferred to cells to produce final drug concentrations of 0.24 μ M to 250 μ M for emodin or 8.23 μ M to 2000 μ M for atanyl blue PRL, acid blue 40, and alizarin violet R. Triplicates of no drug and no cell controls were included on each plate.

After incubation for 5 days, 100 μ l of supernatants were removed and 100 μ l CellTiter-Glo assay reagent (Promega) was added to each well. Luminescence was measured in RLU's using Biotek Synergy HT Multimode Microplate reader and was normalized to “percent maximum luminescence” for easier comparability between experiments. The data were fitted with a nonlinear variable slope four-parameter regression curve and the median toxic dose (TD_{50}) (compound concentration at which luminescence signal was reduced by half) of the compounds was determined using Prism software by GraphPad, Inc.

GFP-Based Assay of Entry and GFP Expression

Clear-walled, clear-bottom 96-well plates containing confluent monolayers of MRC-5 cells were infected with BADrUL131-Y4 at 10,000 pfu (plaque forming units) per well (MOI=1). Six three-fold dilutions of atanyl blue PRL were prepared in 200 μ l MEM in quadruplicate in a 96-well format. The atanyl blue PRL dilutions were transferred to cells immediately after virus infection to produce final compound concentrations of 1.03 to 250 μ M. Photomicrographs of GFP fluorescence were taken every two days using a Nikon Eclipse TS100 inverted microscope and GFP fluorescence was quantitated using a Biotek Synergy HT Multimode Microplate Reader. The data were normalized by converting RLU's to "percent maximum fluorescence" for each experiment. The data were fitted with a nonlinear variable slope four-parameter regression curve and the half-maximal inhibitory concentrations (IC_{50}) (drug concentrations at which fluorescence signal was reduced by half) of the compounds were determined using Prism software by GraphPad, Inc. Photomicrographs were organized by day and concentration of compound using Illustrator by Adobe Systems Incorporated.

GFP-Based Assay of Viral Spread

Clear walled, clear bottom 96-well plates containing confluent monolayers of MRC-5 cells were infected with BADrUL131-Y4 at 1,000 pfu (MOI=0.1) or 100 pfu (MOI=0.01) per well. Six three-fold dilutions of atanyl blue PRL were prepared in 200 μ l MEM in quadruplicate in a 96-well format. The atanyl blue PRL dilutions were transferred to cells immediately after virus infection to produce final compound concentration of 1.03 to 250 μ M. Every two days, photomicrographs of GFP fluorescence were taken using a Nikon Eclipse TS100 inverted microscope and RLU's were measured using Biotek Synergy HT Multimode Microplate Reader.

The data were normalized by converting RLU's to "percent maximum fluorescence" for each experiment. The data from the 1,000 pfu per well infection were fitted with a nonlinear variable slope four-parameter regression curve and the IC₅₀ (drug concentrations at which fluorescence signal was reduced by half) of the compounds were determined using Prism software by GraphPad, Inc. Using the photomicrographs from the 100 pfu per well infection, a single infected cell was examined over time for viral spread and growth to neighboring cells. Photomicrographs were organized by day and concentration of compound using Illustrator by Adobe Systems Incorporated.

GFP-Based Infectivity Assay for Susceptibility of CMV to Atanyl Blue PRL

Two clear-walled, clear-bottom 96-well plates containing confluent monolayers of MRC-5 cells were infected with BADrUL131-Y4 (MOI=0.01). Five three-fold dilutions of atanyl blue PRL were prepared in 200 µl MEM. These compound dilutions were transferred to infected cells with 32 wells for each dilution to produce final drug concentrations of 3.09 µM to 250 µM. 32 wells of no drug control were also included on the plate. After incubation for 14 days, each of the six sets of 32 wells was counted for the presence or lack of green fluorescence using a Nikon Eclipse TS100 inverted microscope. The presence of fluorescence indicated a positive well. The number of positive wells out of 32 was converted to "percent positive wells" and was fitted with a nonlinear variable slope four-parameter regression curve and the IC₅₀ (amount of compound concentration at which 50% of no drug control were positive for fluorescence) of the compounds was determined using Prism software by GraphPad, Inc.

Time of Addition Assay

Clear-walled, clear-bottom 96-well plates containing confluent monolayers of MRC-5 cells were infected with RC2626 virus (MOI=0.03). Atanyl blue PRL was prepared at a concentration of 27.8 μ M in 200 μ l MEM and added in triplicates at 0 hours, 1 hour, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, or 120 hours post infection. Triplicates of ganciclovir (10 μ M), BAY 38-4766 (8 μ M), no drug, and no virus were also included on the plate. In a second group triplicate samples of RC2626 were incubated with atanyl blue PRL at 27.8 μ M for 1 hour followed by 10-fold dilution with culture medium. Then added to cells (MOI=0.03).

After incubation for 5 days, 50 μ l supernatants from the 96-well plate were transferred to a black-walled, clear-bottom 96-well plate containing confluent MRC-5 monolayers. After 24 hours, 100 μ l Steady-Glo luciferase assay reagent (Promega Corporation) was added. After allowing 10 minutes for the luciferase assay reagent to lyse the cells, the luciferase activity was measured in relative light units (RLU's) using Biotek Synergy HT Multimode Microplate Reader. To allow easier comparability between experiments, the data was normalized by converting RLU's to "percent maximum luminescence" for each experiment. In order to visualize the effect of the compound when added at different times after infection, percent maximum luminescence was plotted as a function of time using Prism software by GraphPad, Inc.

Western Immunoblot Analysis Assay

T25 flasks (Corning) containing confluent MRC-5 cells were infected with BadrUL131-Y4 at 8×10^5 pfu/flask (MOI=0.8). Compounds were added immediately after infection and cells

were harvested after 24, 48, 72, and 96 hours along with untreated cultures at the same time points and a non-infected control. Whole cell lysates were collected by scraping into media and pelleted by centrifugation for 5 minutes at 2000 rpm. Pelleted cells were lysed in Laemmli buffer (Bio-Rad) and beta-mercaptoethanol was added. Lysates were vortexed, heated to 95 degrees Celsius for 5 minutes, then centrifuged for 3 minutes at 6000 rpm. Supernatants were aliquoted and stored at -20 degrees Celsius. Prior to loading on the gel lysates were heated to 95 degrees Celsius for 5 minutes. Lysates were separated on 10-20% Criterion Tris-HCl polyacrylamide gels (Bio-Rad) at 100 volts for 2 hours in a 1x Tris/glycine/SDS running buffer (Bio-Rad). Separated proteins were transferred to 0.4 µm nitrocellulose membranes (Bio-Rad) at 0.42 amps for 2 hours in a 10% 10X Tris/glycine (Bio-Rad) and 20% methanol transfer buffer. Membranes were blocked for 1 hour in phosphate buffered saline (PBS), 0.02% Tween-20 (Fisher), and 5% powdered milk. Primary antibodies used were anti-cytomegalovirus mouse monoclonal antibody to UL98 (I-2-gift from Jay Nelson), mouse monoclonal to UL99 (Goodwin Institute), mouse monoclonal antibody to IE1/2 mAB810 (Millipore), and rabbit polyclonal antibody to glycoprotein B (Vical) and were diluted in blocking buffer at 1:1000, 1:1000, 1:500, and 1:2000 dilutions respectively. Membranes were incubated with primary antibody at 4 degrees Celsius overnight. Membranes were washed every 5 minutes for 30 minutes with a 0.02% Tween-20/PBS wash buffer. Secondary antibodies used were horseradish peroxidase (HRP)-conjugated goat anti-mouse antibody (Thermo-Fisher) used for mouse primary antibodies and goat anti-rabbit antibody (Thermo-Fisher) used for rabbit primary antibodies. Secondary antibodies were diluted in blocking buffer at 1:5000 dilution and incubated for 1 hour at room temperature with agitation on a rocker. The membranes were washed every 5 minutes for 30 minutes and incubated for 5 minutes with enhanced chemiluminescence western blotting substrate (Thermo-

Fisher). Chemiluminescence was detected by exposure of X-ray film (Thermo-Fisher) at various times of exposure and processed using Kodak X-Omat 1000A processor.

III. RESULTS

A. Evaluation of Potential UL98 Inhibitor Compounds

1. Introduction

The protein UL98 is an alkaline nuclease encoded by the *UL98* gene of CMV. Herpesvirus alkaline nucleases have exonuclease and endonuclease activity and may be involved in viral DNA processing and capsid egress from the nucleus (29). It has been shown that *UL98* null mutants are viable; however, viral replication is attenuated by up to 1,000-fold, indicating that while UL98 is not an essential CMV gene, it is highly important for efficient replication (50). Due to the importance of UL98 in CMV replication, investigation of UL98 could lead to a novel target for the development of new CMV antivirals.

Our collaborators Hardik Parikh and Glenn Kellogg (VCU Institute for Structural Biology and Drug Discovery) used the amino acid sequence of CMV UL98 alkaline nuclease (strain AD169) to identify a template structure based on a model structure for UL98 could be built. The Shutoff and Exonuclease Protein (SOX) from Kaposi's sarcoma-associated herpesvirus (KSHV) was chosen as the template structure for the model building of CMV UL98. The amino acid sequence of UL98 was aligned to the sequence of KSHV-SOX, and the active site and 5' phosphate binding residues in UL98 alkaline nuclease were identified based on their alignment with the corresponding residues in KSHV-SOX. The predicted active site amino acids R164, S252, D254, E278, and K280, are shown in figure 6. A homology model of UL98 alkaline nuclease was built based on the crystal structure of KSHV-SOX and the sequence alignment. A model was

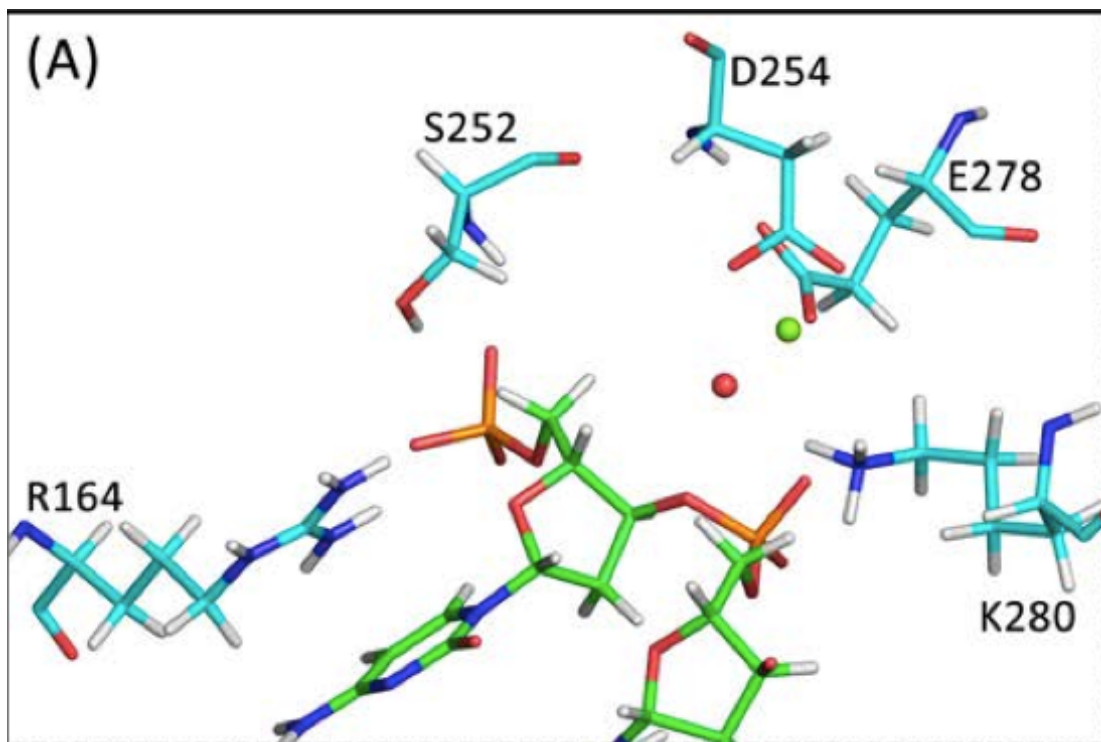


Figure 6: Active site residues of UL98 alkaline nuclease. The predicted active site amino acids of CMV UL98 alkaline nuclease shown are R164, S252, D254, E278, and K280. Figure courtesy of Hardik Parikh and Glenn Kellogg.

selected based on its ability to accommodate a DNA fragment in the active site as well as satisfying spatial, carbon-carbon and nitrogen-oxygen distance, and bond angle constraints (50). The UL98 alkaline nuclease-double stranded DNA complex was used to identify important molecular interactions in that association, which allowed a model to be generated that showed desirable ligands that might bind at the active site of UL98 and inhibit its activity. A 3-dimensional model was created that was used to perform a virtual screening of the National Cancer Institute Open Database of over 250,000 compounds in order to find potential UL98 inhibitor compounds. Each potential UL98 inhibitor compound was docked, *in silico*, to the UL98 alkaline nuclease active site model and ranked based on binding free energy. The compounds were also analyzed visually to verify reasonable binding in the active site such that the catalytically important residues would be involved. Using this procedure, our collaborators were able to use the homology model of UL98 to conduct a virtual screening to identify potential inhibitors of UL98's alkaline nuclease activity; the 15 compounds most likely to inhibit UL98 activity are shown in figure 7.

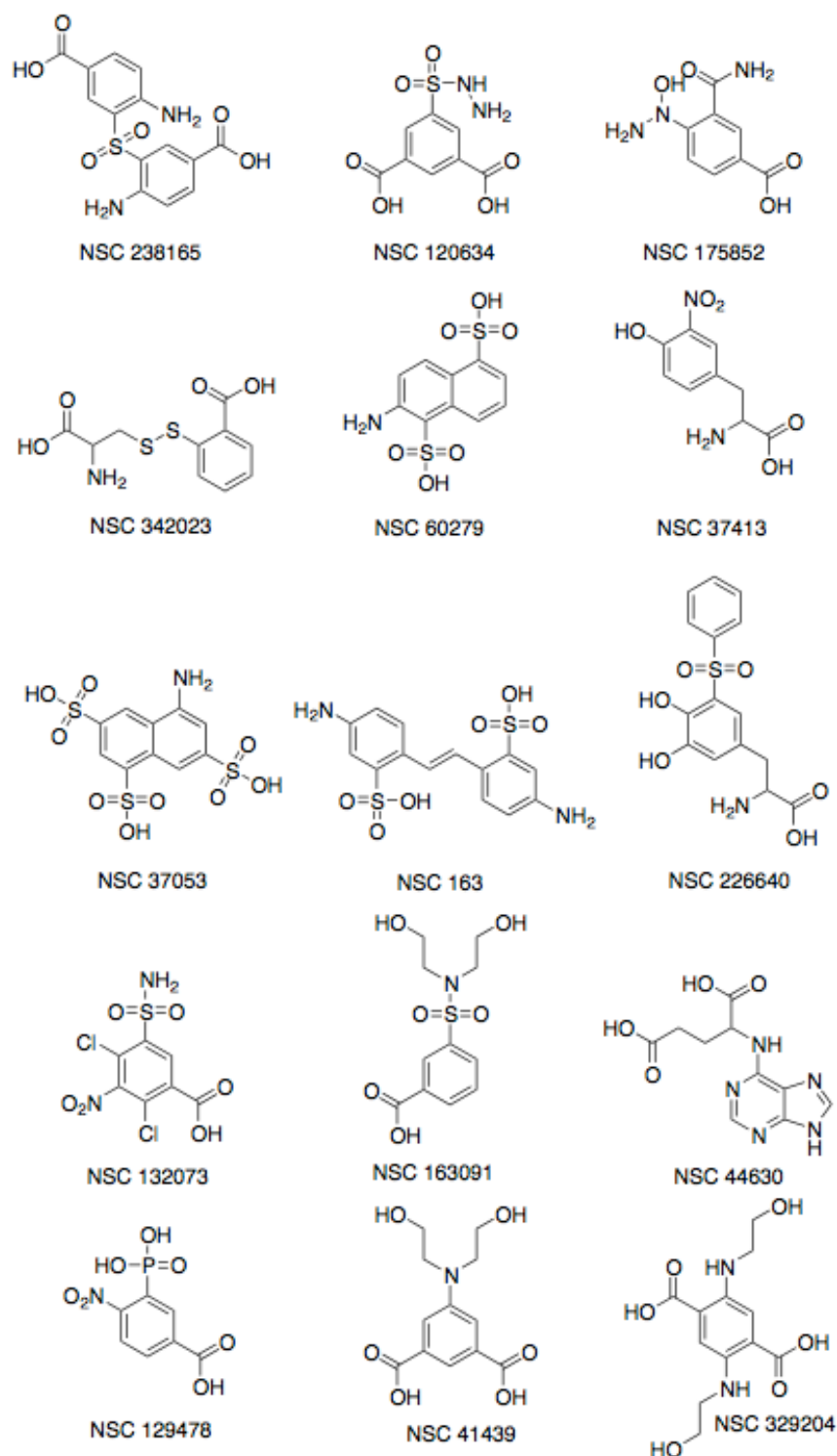


Figure 7: Top 15 compounds most likely to inhibit UL98 alkaline nuclease activity. Compound structures shown above are the 15 compounds from the National Cancer Institute Open Database compounds that are most likely to inhibit UL98 alkaline nuclease activity based on *in silico* screening. Unpublished data courtesy of Hardik Parikh and Glenn Kellogg.

2. Results

In order to investigate the antiviral activity of the 15 potential UL98 inhibitor compounds from the National Cancer Institute, 96-well plates containing MRC-5 cells were infected with RC2626 virus incubated for one hour. Potential UL98 inhibitors were added at 25 μ M and 100 μ M final concentrations. After incubation for five days, the amount of infectious virus in the culture supernatants was measured by transferring 50 μ l of supernatants to a black-walled, clear-bottom 96-well plate containing MRC-5 monolayers. After 24 hours, luciferase activity was measured.

Figure 8 shows the luminescence of the 15 potential UL98 inhibitor compounds at 100 μ M concentration. Also shown are the no-drug, no-virus, and ganciclovir controls. The 15 compounds displayed a luciferase signal of approximately 60,000 to 70,000, which was very similar to the no-drug control. The ganciclovir control reduced the luciferase signal down to almost the same level as the no-virus control.

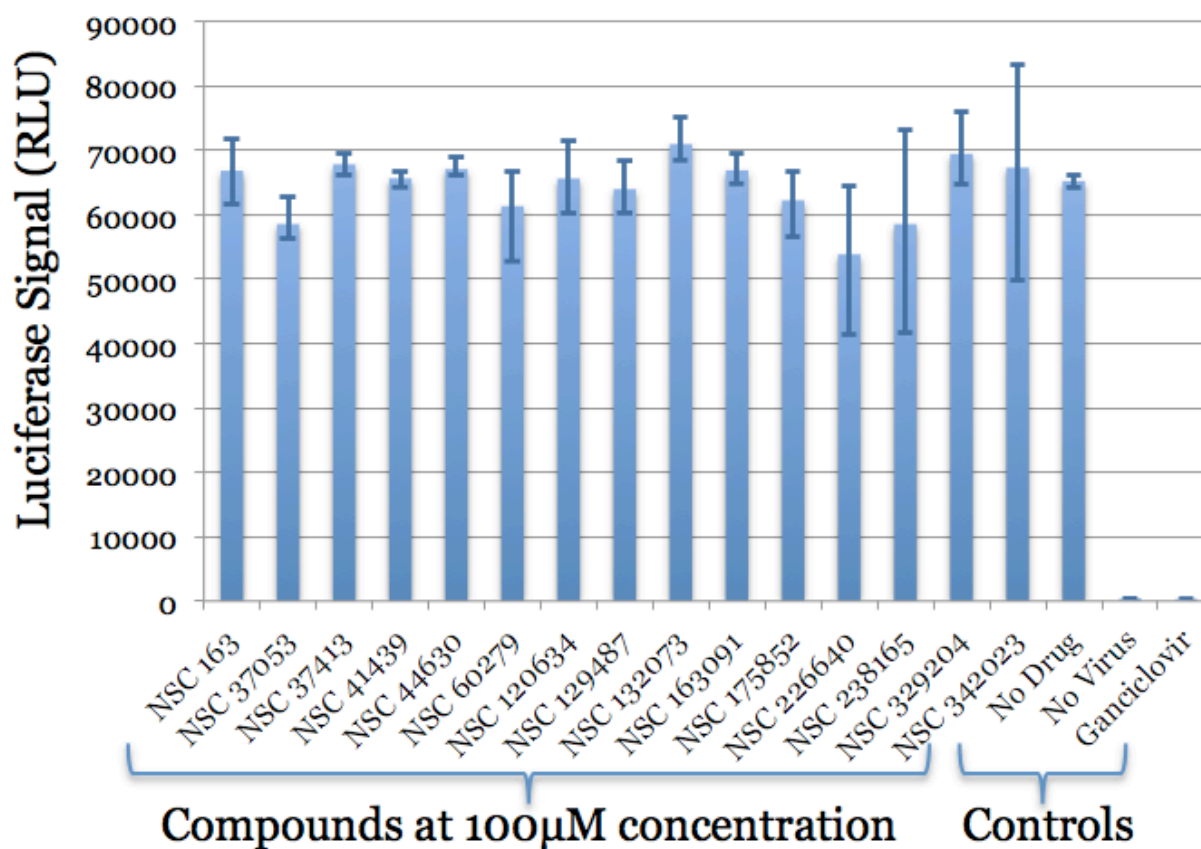


Figure 8: Luciferase-based antiviral activity of 15 potential UL98 inhibitors from National Cancer Institute. Confluent MRC-5 monolayers in 96-well plates were infected with RC2626 (MOI = 0.03) and incubated for 5 days in the presence of different compounds at a concentration of 100 μ M. Supernatants were transferred to another 96-well plate with MRC5 monolayers, incubated for 24 hours, and luciferase signal was measured.

3. Conclusions

The 15 potential UL98 inhibitor compounds did not significantly reduce the luciferase signal even at a very high concentration of 100 μ M, suggesting that the compounds did not have any antiviral activity. However, these compounds may still have activity against UL98 alkaline nuclease but were unable to cross the cell membrane, and therefore were unable to exhibit antiviral activity. An evaluation of activity against UL98 nuclease activity *in vitro* is planned which will address the concern of the potential UL98 inhibitor compounds being unable to cross the cell membrane.

B. Evaluation of Emodin, Atanyl Blue PRL, Acid Blue 40, and Alizarin Violet R

1. Introduction

Anthraquinones are aromatic organic compounds with the chemical formula $C_{14}H_8O_2$ (figure 4). Anthraquinones are present in many plant extracts such as *Rheum officinale*, *Aloe barbadensis*, *Rhamnus frangula*, *Rhamnus purshianus*, *Cassia angustifolia*, *Hypericum perforatum*, and *Polygonum genera* (22, 28). It has been previously described that emodin, an anthraquinone derivative, specifically inhibits the nuclease activity of the HSV-1 alkaline nuclease, UL12 (22). It has also been reported that emodin demonstrated antiviral activity not only against herpes simplex virus type 1 but also herpes simplex virus type 2 both *in vitro* and *in vivo* (29).

Further, it has also been described that anthraquinone derivatives have antiviral activity against CMV (24). Several anthraquinone derivatives showed activity against CMV, including emodin, atanyl Blue PRL, acid blue 40, and alizarin violet R; however, their mechanism of anti-CMV activity has not been explored (23, 24). These findings

suggest that anthraquinone derivatives could be used as alternative antiviral agents against CMV. Moreover, since alkaline nucleases are highly conserved throughout the herpesvirus family, it is important to determine if anthraquinone derivatives act to inhibit CMV through inhibition of UL98 alkaline nuclease (50).

2. Results

To explore the antiviral activity of emodin, atanyl blue PRL, acid blue 40, and alizarin violet R, the same luciferase-based assay was used as in the previous section except a range of concentrations of the test compounds were tested.

To explore the cytotoxicity of emodin, atanyl blue PRL, acid blue 40, and alizarin violet R, 96-well plates containing monolayers of MRC-5 cells were incubated with dilutions of the test compounds in 200 μ l MEM in a 96-well format along with triplicates of no drug and no cell controls.

After incubation for 5 days, 100 μ l of supernatants were removed and 100 μ l CellTiter-Glo assay reagent was added to each well. The luciferase signal is proportional to ATP present in the cell cultures, which reflects the number of viable cells. Luminescence was measured and the median toxic dose (TD_{50}) of the compounds was determined.

Figure 9 shows the antiviral activity and toxicity of emodin. Emodin was used at concentrations of 0.24 μ M to 250 μ M and the nonlinear regression curve showed antiviral activity with an IC_{50} of 4.9 μ M and cytotoxicity with a TC_{50} of 9.2 μ M. The cytotoxicity is approximately two-fold higher than the antiviral activity. Figure 10 shows the antiviral activity for atanyl blue PRL, acid blue 40, and alizarin violet R. These

compounds were used at concentrations of 1.03 μM to 250 μM . The nonlinear regression curve showed antiviral activity of acid blue 40, atanyl blue PRL, and alizarin violet R to have an IC_{50} of 266 μM , 6.3 μM , and 238 μM respectively. Figure 11 shows the cytotoxicity of acid blue 40, atanyl blue PRL, and alizarin violet R. These compounds were used at concentrations of 8.23 μM to 2000 μM . The nonlinear regression curve showed cytotoxicity of acid blue 40, atanyl blue PRL, and alizarin violet R to have a TD_{50} of 231 μM , 216 μM , and 342 μM , respectively. Acid blue 40 and alizarin violet R inhibited CMV only at high concentrations; however, most of their apparent activity appears to be due to cytotoxicity. Lastly, figure 12 shows the antiviral activity and cytotoxicity of atanyl blue PRL side-by-side. Atanyl blue PRL, exhibited anti-CMV activity at an IC_{50} of 6.3 μM with low cytotoxicity with a TD_{50} of 216 μM . Table 1 summarizes the antiviral and cytotoxicity data.

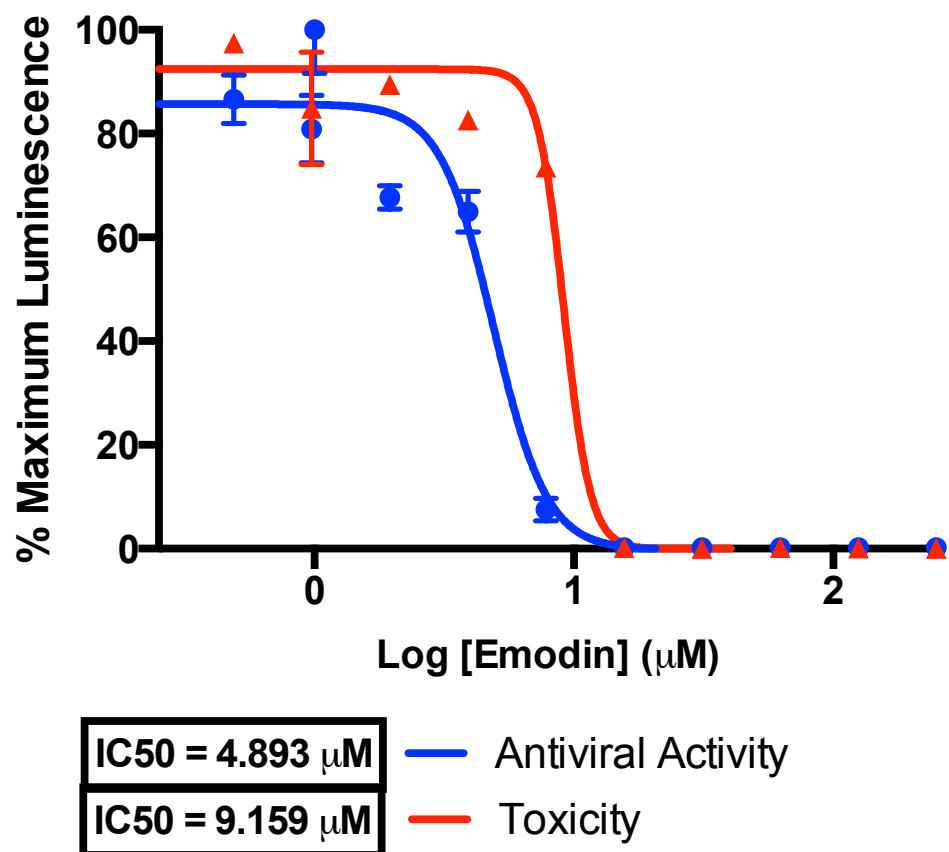


Figure 9: Luciferase-based antiviral activity and cytotoxicity of emodin. For antiviral activity, confluent MRC-5 monolayers in 96-well plates were infected with RC2626 (MOI = 0.03) and incubated for 5 days in the presence of emodin at concentrations of 0.24 μM to 250 μM. Supernatants were transferred to another 96-well plate with MRC-5 monolayers, incubated for 24 hours, and luciferase activity was measured. For cytotoxicity, 96-well plates containing uninfected confluent monolayers of MRC-5 cells were incubated with emodin at concentrations of 0.24 μM to 250 μM. After incubation for 5 days, cell viability was measured using CellTiter-Glo assay reagent.

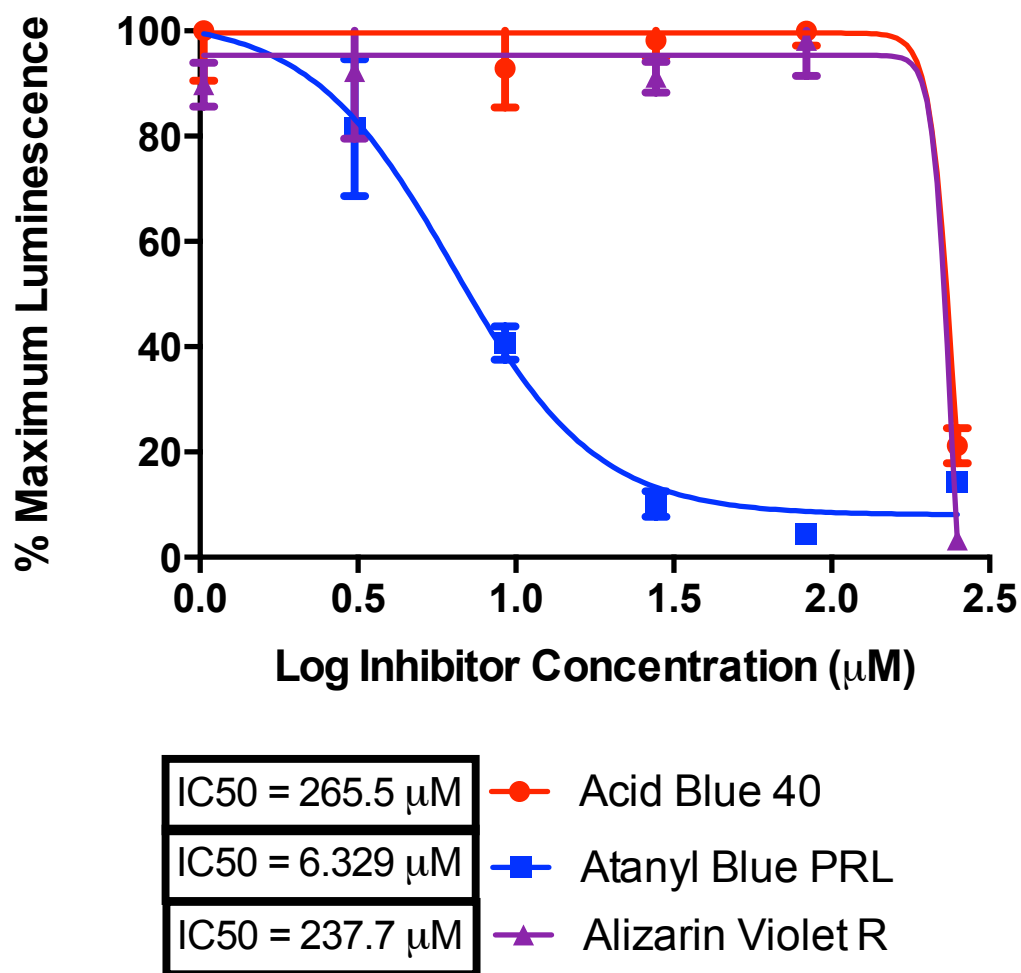


Figure 10: Luciferase-based antiviral activity of acid blue 40, atanyl blue PRL, and alizarin violet R. Confluent MRC-5 monolayers in 96-well plates were infected with RC2626 (MOI = 0.03) and incubated for 5 days in the presence of acid blue 40, atanyl blue PRL, and alizarin violet R at a concentration of 0.24 μM to 250 μM. Supernatants were transferred to another 96-well plate with MRC-5 monolayers, incubated for 24 hours, and luciferase activity was measured.

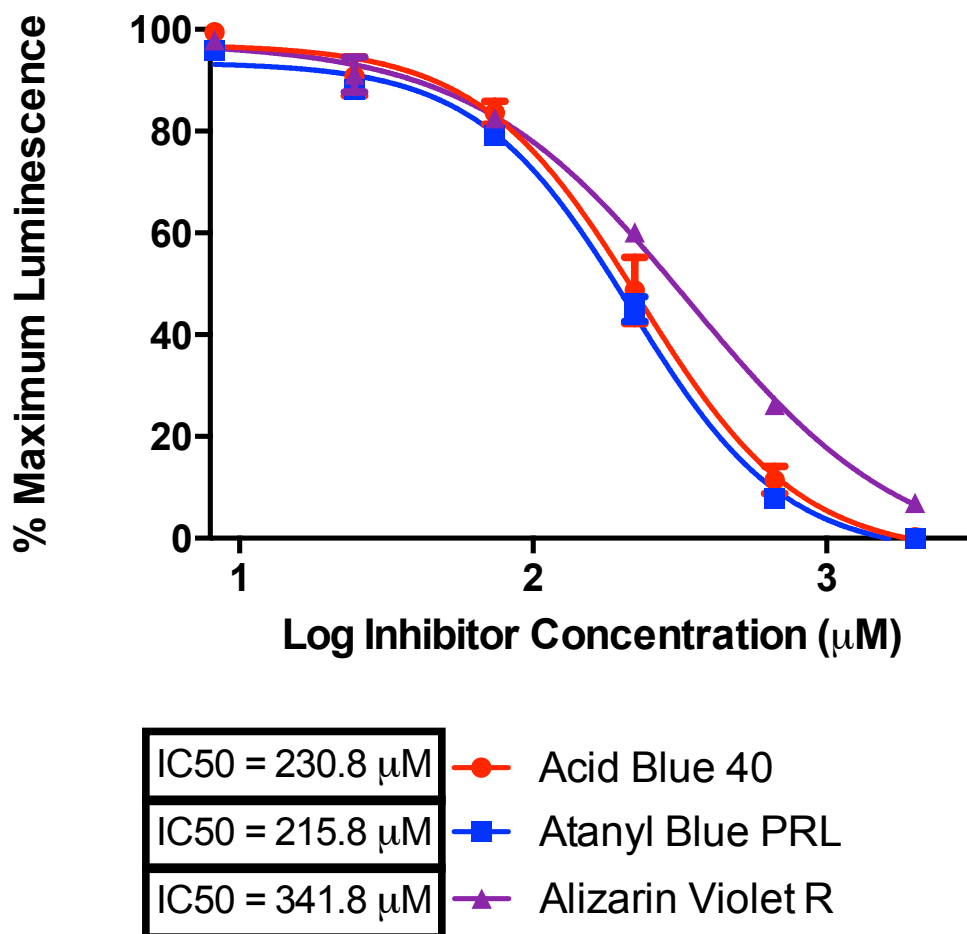


Figure 11: Luciferase-based cytotoxicity of acid blue 40, atanyl blue PRL, and alizarin violet R. 96-well plates containing uninfected confluent monolayers of MRC-5 cells were incubated with acid blue 40, atanyl blue PRL, and alizarin violet R at concentrations of 8.23 μM to 2000 μM. After incubation for 5 days, cell viability was measured using CellTiter-Glo assay reagent.

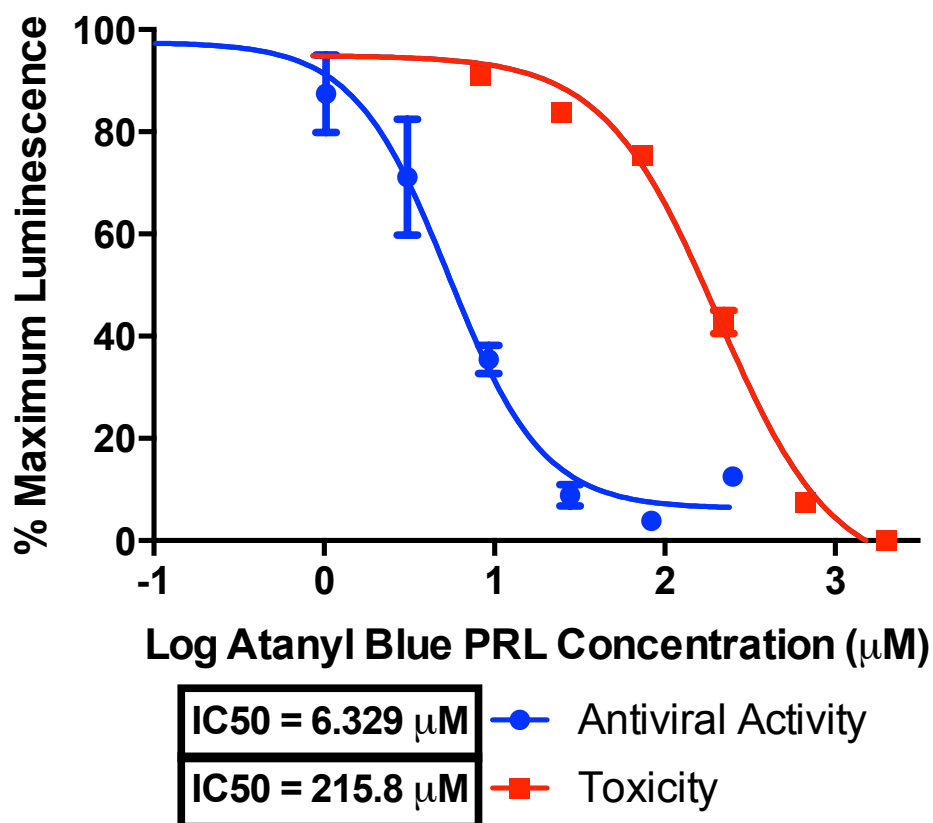


Figure 12: Luciferase-based antiviral activity and cytotoxicity of atanyl blue PRL. For antiviral activity, confluent MRC-5 monolayers in 96-well plates were infected with RC2626 (MOI = 0.03) and incubated for 5 days in the presence of atanyl blue PRL at concentrations of 0.24 μM to 250 μM . Supernatants were transferred to another 96-well plate with MRC-5 monolayers, incubated for 24 hours, and luciferase activity was measured. For cytotoxicity, 96-well plates containing uninfected confluent monolayers of MRC-5 cells were incubated with atanyl blue PRL at concentrations of 8.23 μM to 2000 μM . After incubation for 5 days, cell viability was measured using CellTiter-Glo assay reagent.

Anthraquinone Compound	Antiviral Activity (IC₅₀)	Cytotoxicity (TD₅₀)
Emodin	4.9 μ M	9.2 μ M
Acid Blue 40	266 μ M	231 μ M
Atanyl Blue PRL	6.3 μ M	216 μ M
Alizarin Violet R	238 μ M	342 μ M

Table 1: Summary of results from luciferase-based antiviral activity and cytotoxicity assays. Four anthraquinones that were evaluated are shown.

3. Conclusion

Emodin showed antiviral activity with an IC_{50} of 4.9 μM and cytotoxicity with a TD_{50} value of 9.2 μM . Since the cytotoxicity is approximately two-fold higher than the antiviral activity, it suggests that emodin may act non-specifically. Because the antiviral activity and cytotoxicity of emodin were similar, it will not serve as a good compound to investigate further. The antiviral activities of acid blue 40, atanyl blue PRL, and alizarin violet R were found to have an IC_{50} values of 266 μM , 6.3 μM , and 238 μM respectively. The cytotoxicities of acid blue 40, atanyl blue PRL, and alizarin violet R to have TD_{50} values of 231 μM , 216 μM , and 342 μM , respectively. Acid blue 40 and alizarin violet R exhibited anti-CMV activities only at high concentrations nearly matching their cytotoxic levels. Thus, most of their apparent antiviral activity may be due to cytotoxicity. Atanyl blue PRL exhibited anti-CMV activity at an IC_{50} of 6.3 μM and cytotoxicity with a TD_{50} of 216 μM ; a difference of approximately 1.5 logs between cytotoxicity and antiviral activity. Since atanyl blue PRL displayed anti-CMV activity with low cytotoxicity, the antiviral activity appears to be specific. Therefore, atanyl blue PRL is a useful compound to investigate further and characterize the mechanism of action.

C. Evaluation of Atanyl Blue PRL

1. Introduction

It has been reported that atanyl blue PRL has a anti-CMV activity with an IC_{50} of 7 μM and a TD_{50} value of 275 μM (31). Our data indicates similar results that the anthraquinone derivative, atanyl blue PRL, shows anti-CMV activity ($IC_{50} = 6.3 \mu M$) with low cytotoxicity ($TD_{50} = 216 \mu M$). However, questions remain regarding the

mechanism of action of this compound. Characterization of atanyl blue PRL, including impact on gene expression, GFP expression, impact on viral cell entry, impact on viral spread, impact on infectivity, and time of addition studies should be informative. The related compound emodin has been proposed to inhibit HSV-1 replication through inhibition of the HSV alkaline nuclease UL12. It has been demonstrated that *UL98* is an important CMV gene, so demonstration that atanyl blue PRL inhibits CMV through inhibition of UL98 alkaline nuclease would serve to validate UL98 as a novel antiviral target and provide a lead structure for structure-based drug discovery (50).

2. Results

To evaluate the effect of atanyl blue PRL on GFP expression, 96-well plates containing confluent monolayers of MRC-5 cells were infected with BAD γ UL131-Y4, a virus engineered to express GFP. Atanyl blue PRL dilutions were transferred to cells immediately after infection to produce final compound concentrations of 1.03 μ M to 250 μ M. Photomicrographs of GFP fluorescence were taken and GFP fluorescence was measured every two days.

Figure 13 shows photomicrographs of the effect of atanyl blue PRL on GFP expression. There is a marked reduction in GFP expression with the addition of atanyl blue PRL. There is a significant reduction in GFP expression at concentrations greater than 9.36 μ M. Figure 14 shows the GFP-based dose response of atanyl blue PRL on day 4. The IC₅₀ was determined to be 6.2 μ M, indicating a similar dose response as the luciferase-based antiviral effect.

To evaluate the effect of atanyl blue PRL on viral spread, 96-well plates containing confluent monolayers of MRC-5 cells were infected with BADrUL131-Y4 at 1,000 pfu (MOI=0.1) and 100 pfu (MOI=0.01) per well. The atanyl blue PRL dilutions were transferred to cells immediately after virus to produce final compound concentrations of 1.03 μ M to 250 μ M. Photomicrographs were taken and GFP fluorescence was measured every two days.

Using the photomicrographs from the 100 pfu per well infection, a single infected cell was examined over time for viral spread and growth to neighboring cells. Photomicrographs were organized by day and concentration of compound. Figure 15 shows photomicrographs of the effect of atanyl blue PRL on viral spread. Atanyl blue PRL was used at concentrations of 1.03 μ M to 250 μ M, however, no inhibitor and atanyl blue PRL at 9.26 μ M are shown for day 6, 8, 10, and 12. It can be seen that there is a marked inhibition in viral spread as the days progress with the addition of atanyl blue PRL compared to no inhibitor.

The GFP fluorescence RLUs were quantified, normalized, and fitted to a nonlinear regression curve to determine the IC₅₀ of atanyl blue PRL inhibition. Figure 16 shows the GFP-based dose response of atanyl blue PRL. The IC₅₀ was determined to be 6.3 μ M on day 20, indicating a similar dose response with viral spread as the luciferase-based antiviral effect.

To assess the effect of atanyl blue PRL on the infectivity of CMV virus, 96-well plates containing confluent monolayers of MRC-5 cells were infected with BADrUL131-Y4 at a dilution estimated to result in infection of only approximately 75% of wells. Atanyl blue PRL dilutions were transferred to infected cells with 32 replicates wells for

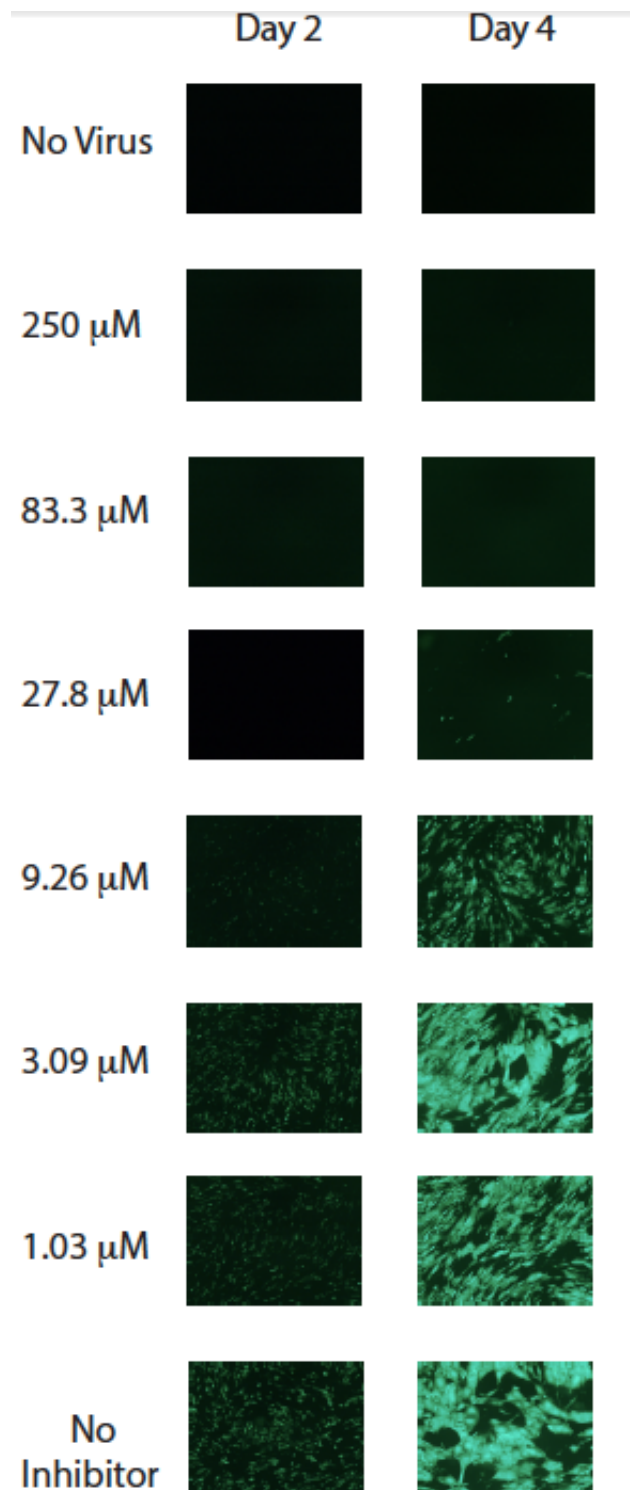


Figure 13: Photomicrographs of the effect of atanyl blue PRL on GFP expression. 96-well plates containing confluent monolayers of MRC-5 cells were infected with BADrUL131-Y4 at an MOI of 1. Atanyl blue PRL dilutions were added to cell at concentrations of 1.03 μ M to 250 μ M. Photomicrographs shown were taken on day 2 and day 4 post infection.

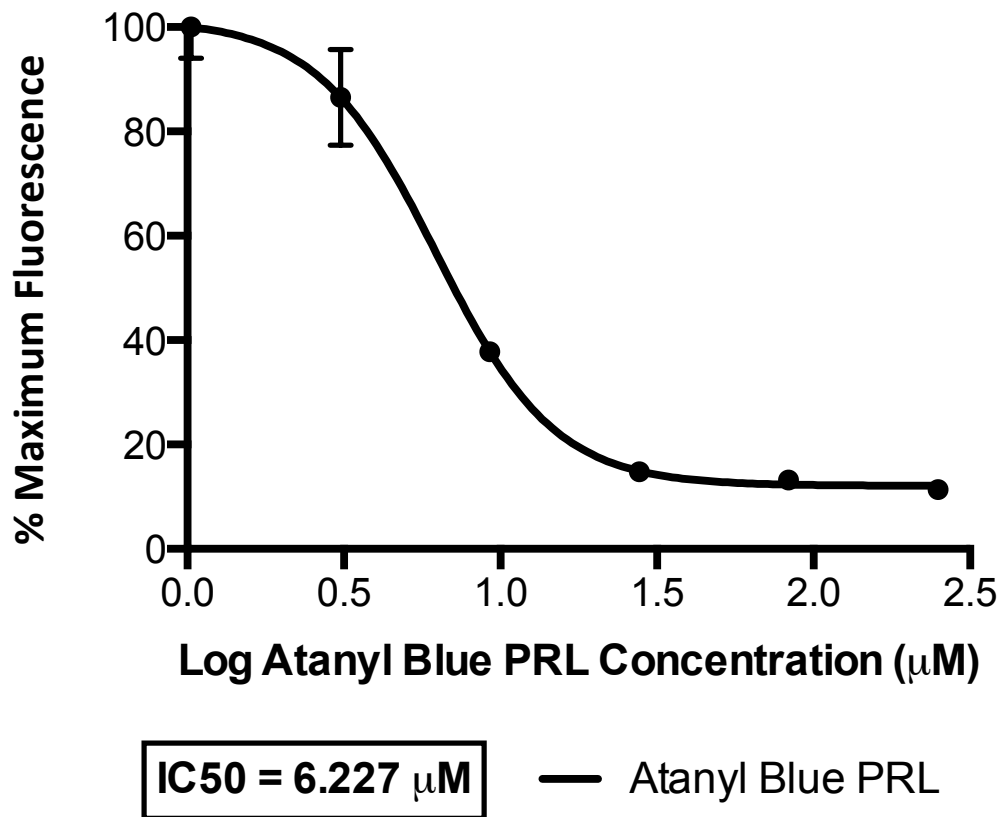


Figure 14: Dose response of atanyl blue PRL. 96-well plates containing confluent monolayers of MRC-5 cells were infected with BADrUL131-Y4 at an MOI of 1. Atanyl blue PRL dilutions were transferred to cells immediately after virus to produce final compound concentrations of 1.03 μM to 250 μM. RLU's of GFP fluorescence were measured four days post infection and normalized to percent maximum fluorescence. The data was normalized by converting RLU's to "percent maximum fluorescence" for each experiment.

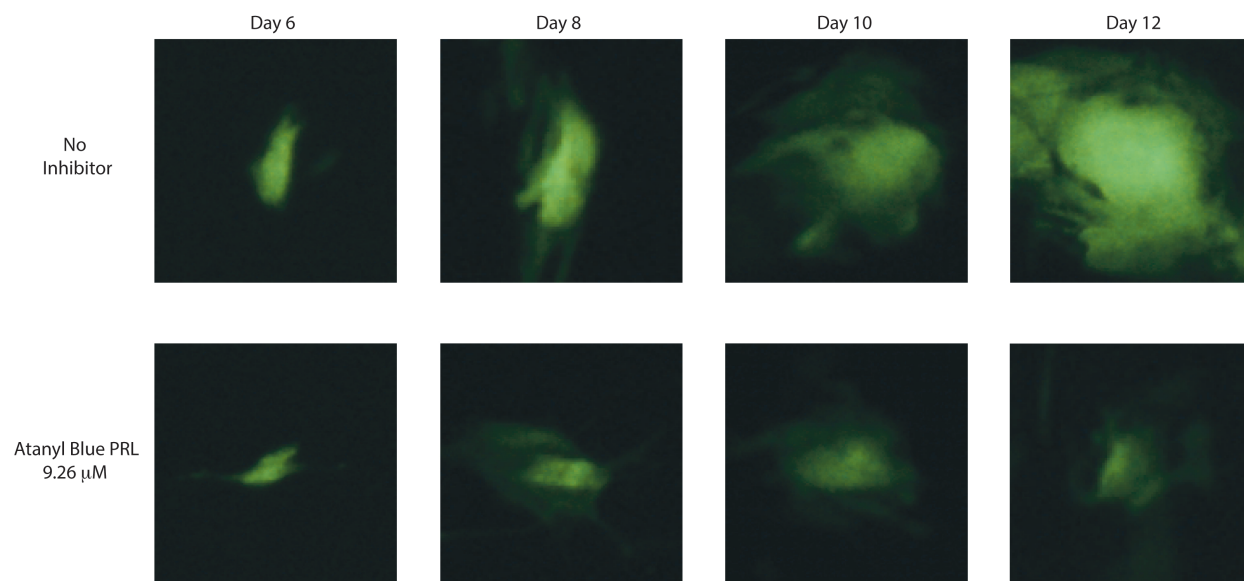


Figure 15: Photomicrographs of the effect of atanyl blue PRL on viral spread. 96-well plates containing confluent monolayers of MRC-5 cells were infected with BADrUL131-Y4 at 100 pfu per well with or without 9.26 μ M atanyl blue PRL. GFP-positive cells were marked on day 6 post infection and the same region of the culture was photographed at two-day intervals.

each concentration to produce final drug concentrations of 3.09 μM to 250 μM . 32 wells of no inhibitor control were also included on the plate. After incubation for 14 days, each of the six sets of 32 wells was scored for the presence or lack of green fluorescence. The presence of fluorescence indicated a positive, virus-infected, well. The number of positive wells out of 32 was converted to “percent GFP positive wells” for each compound dilution and was fitted to a non-linear regression curve and the IC_{50} of the compound was determined.

Figure 17 shows the effect of atanyl blue PRL on infectivity of CMV. Atanyl blue PRL was used at concentrations of 3.09 μM to 250 μM . It can be seen that as the concentration of atanyl blue PRL increases, there is a reduction in the percent of GFP positive wells. The results suggest that atanyl blue PRL lowers the infectivity of the virus, and may cause infected cells to become abortive and unable to produce progeny virus that can infect other cells. The percent GFP-positive wells were normalized and were fitted to a nonlinear regression curve to determine the IC_{50} of atanyl blue PRL. The IC_{50} was determined to be 7.1 μM , indicating a similar dose response as the luciferase-based spread, and GFP-expression assays.

To study the effect of time of addition of atanyl blue PRL after infection, 96-well plates containing confluent monolayers of MRC-5 cells were infected with RC2626 virus ($\text{MOI}=0.03$). Atanyl blue PRL was prepared at a concentration of 27.8 μM in 200 μl MEM and added in triplicates at 0 hours, 1 hour, 6 hours, 12 hours, 24 hours, 48 hours, 72 hours, 96 hours, and 120 hours post infection. Triplicates of ganciclovir (as an early acting control), BAY 38-4766 (as a late acting control), no drug, and no virus were also included on the plate. To determine if preincubation of the compound with the virus has

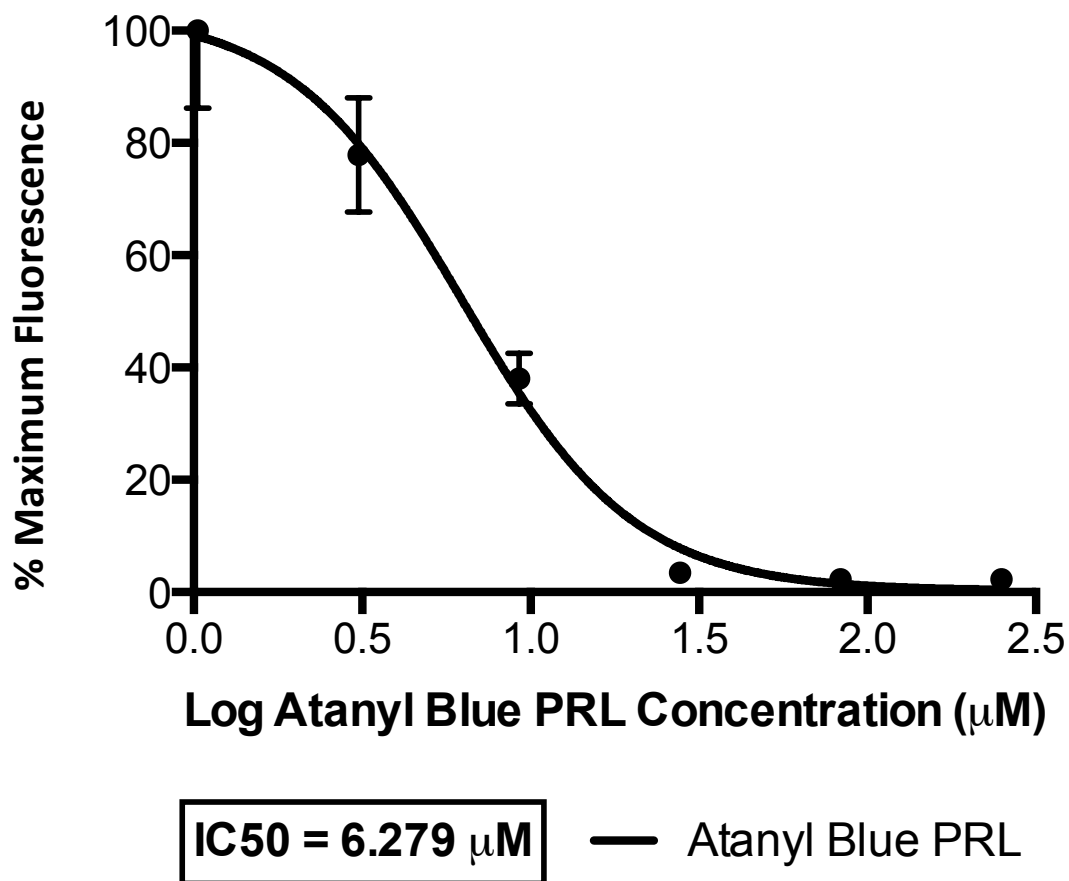


Figure 16: Dose response of atanyl blue PRL (day 20). 96-well plates containing confluent monolayers of MRC-5 cells were infected with BADrUL131-Y4 at 1,000 pfu per well (MOI=0.1). Atanyl blue PRL dilutions were transferred to cells immediately after virus to produce final compound concentrations of 1.03 μM to 250 μM. RLU's of GFP fluorescence were measured every two days. The data was normalized by converting RLU's to "percent maximum fluorescence" for each experiment.

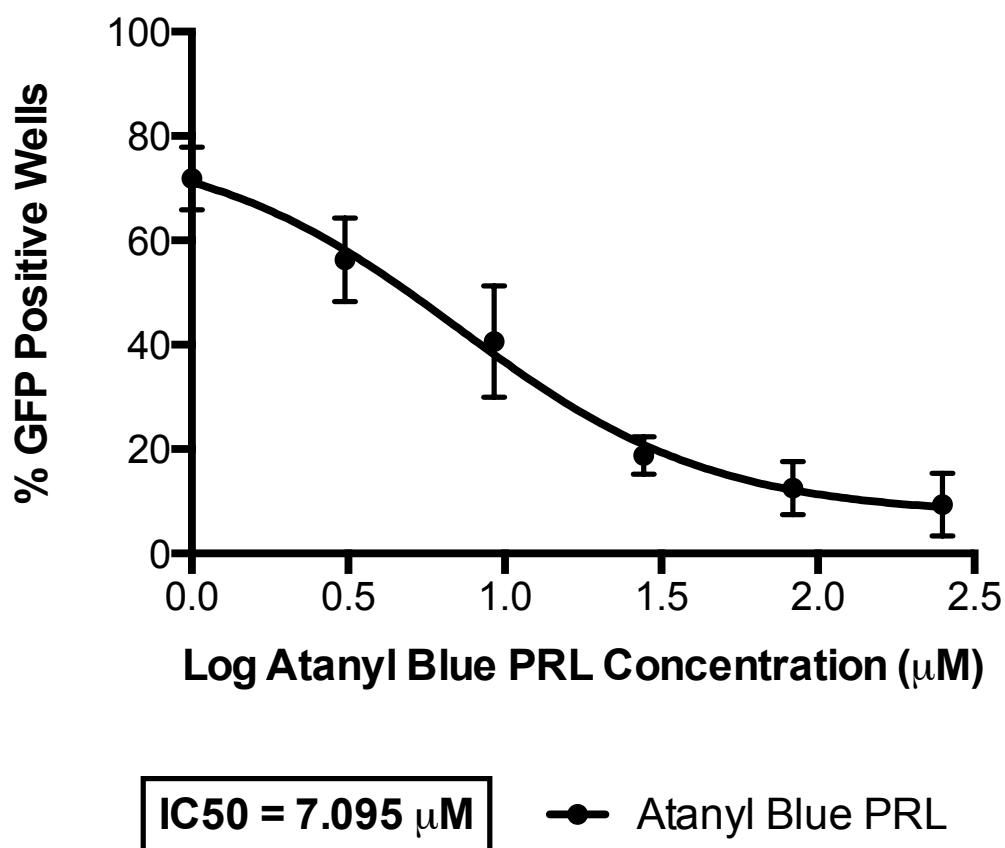


Figure 17: Effect of atanyl blue PRL on infectivity of CMV. 96-well plates containing confluent monolayers of MRC-5 cells were infected with BADrUL131-Y4. Each atanyl blue PRL dilution was added to 32 replicate wells at concentrations of 3.09 μM to 250 μM. After incubation for 14 days, wells were scored for the presence or lack of green fluorescence. The number of positive wells out of 32 was converted to “percent GFP positive wells” and was fitted to a non-linear regression curve and the IC₅₀ of the compounds was determined.

an effect, triplicate samples of RC2626 (MOI=0.30) were incubated with 27.8 μ M atanyl blue PRL for 1 hour followed by 10-fold dilution in culture media before addition to cells. After incubation for 5 days, 50 μ l supernatants from the 96-well plate were transferred to another 96-well plate containing confluent MRC-5 monolayers. After 24 hours the luciferase activity was measured in relative light units (RLU's). The data were normalized by converting RLU's to "percent maximum luminescence" for each experiment. In order to visualize the effect of the compound, percent maximum luminescence as a function of time, was plotted.

Figure 18 shows the effect of time of addition of atanyl blue PRL post infection. Atanyl blue PRL was used at concentrations of 27.8 μ M. In panel A of figure 18, atanyl blue PRL was added 0, 24, 48, 72, 96, and 120 hours post infection. The results indicate that atanyl blue PRL is only effective if added before 24 hours post infection. Ganciclovir is only effective if added before 72 hours and BAY 38-4766 is ineffective if added after 72 hours. In panel B of figure 18, atanyl blue PRL was added 0, 1, 6, 12, 24, and 48 hours post infection. The results indicate that atanyl blue PRL is most effective if added before 6 hours post infection, and ineffective if added after 24 hours post infection. This data suggests that atanyl blue PRL is an early acting compound. Pretreatment had no effect, suggesting that atanyl blue PRL does not interact with or modify the virion to alter attachment or entry of virus into cells or that inhibition is reversible.

In order to analyze the effect of atanyl blue PRL on immediate early, early, and late gene expression, western immunoblot analysis was performed. Confluent MRC-5 cells were infected with Badr/UL131-Y4 at 8×10^5 pfu/flask (MOI=0.8). Compounds were added immediately after infection and cells were harvested along with untreated

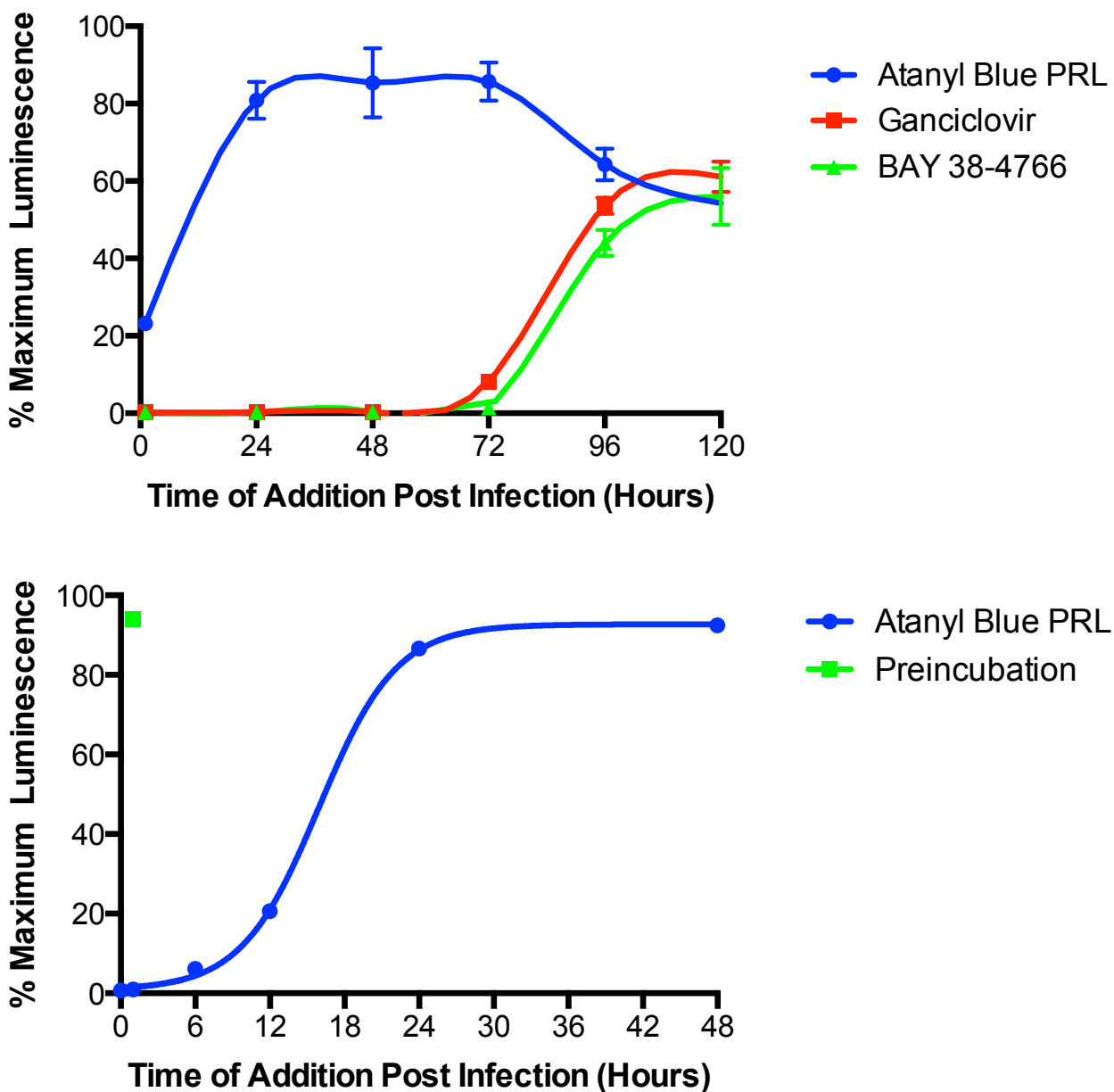


Figure 18: Effect of time of addition of atanyl blue PRL post infection. 96-well plates containing confluent monolayers of MRC-5 cells were infected with RC2626 virus (MOI=0.03). In panel (A), atanyl blue PRL, GCV, or BAY 38-4766 were used at final concentrations of 27.8 μ M, 10 μ M, and 8 μ M respectively and added at 0, 24, 48, 72, 96, or 120 hours post infection. In panel (B) atanyl blue PRL was added at 0, 1, 6, 12, 24, or 48 hours post infection. Preincubation of RC2626 with atanyl blue PRL for 1 hour followed by 10-fold dilution in culture media before addition to cells was added at 0 hours. After incubation for 5 days, 50 μ l supernatants from the 96-well plate were transferred to another 96-well plate containing confluent MRC-5 monolayers and luciferase activity was measured after 24 hours.

control cultures 24, 48, 72, and 96 hours after infection. Lysates were separated on polyacrylamide gels and separated proteins were transferred to nitrocellulose membranes. Specific antibodies were used to detect UL98, UL99, IE1/2, and glycoprotein B.

Figure 19 shows the photomicrographs of the GFP expression of the cultures before harvesting for western immunoblot analysis. Consistent with previous experiments, atanyl blue PRL completely blocked GFP expression, at early times, although some GFP expression occurred at later stages of infection. Figure 20 illustrates the results of western immunoblot analyses. The top panel probes immediate-early protein expression with IE 1/2 mAB810 antibody, the middle panel explores early protein expression with UL98 and UL99 antibodies, and the bottom panel explores late protein expression with glycoprotein B antibody. The results indicate that atanyl blue PRL completely blocks IE1/2 expression on days 1 and 2. Some IE1/2 expression is seen on day 3 and 4, however, it is significantly reduced when compared to the replicate without atanyl blue PRL. Similar results were seen for early protein expression. Atanyl blue PRL reduced UL98 and UL99 expression when compared to the replicates without the inhibitor added. Finally, for late protein expression, atanyl blue PRL reduced the levels of glycoprotein B on days 1, 2, 3, while little difference was observed by day 4.

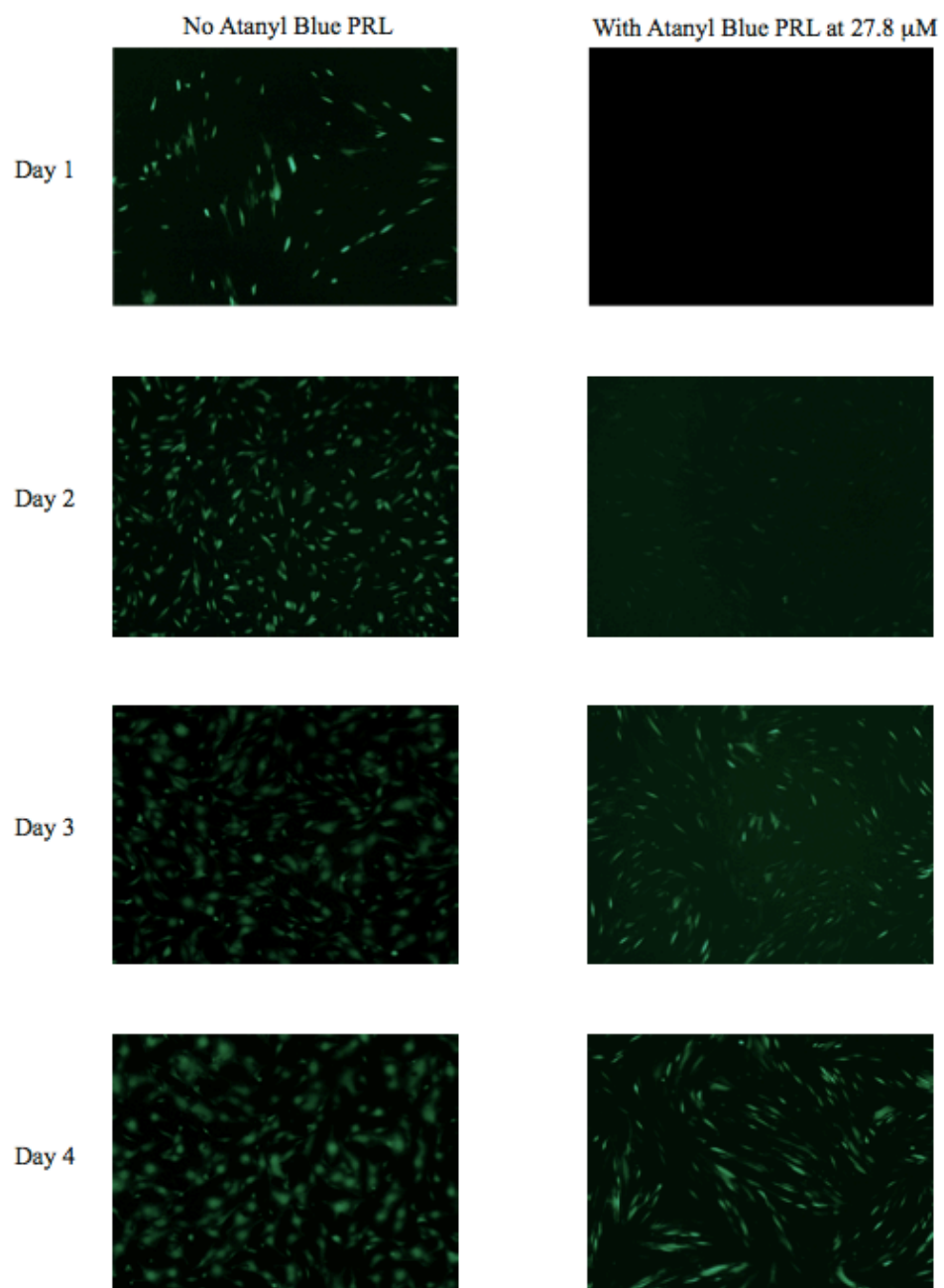


Figure 19: Photomicrographs of GFP expression of BadrUL131-Y4 before harvest for western immunoblot analysis. With and without atanyl blue PRL for days 1, 2, 3, and 4 are shown.

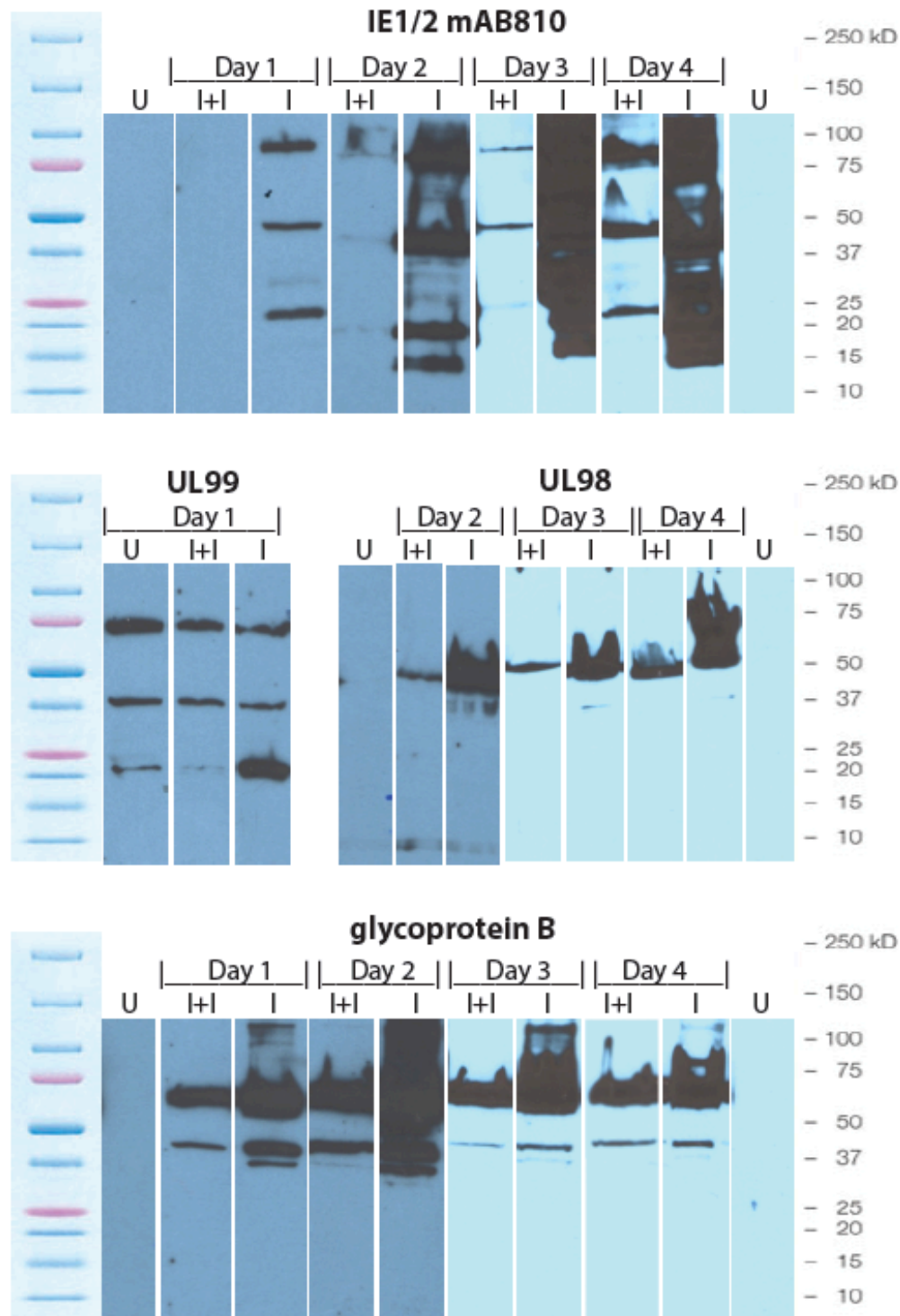


Figure 20: Western immunoblot analysis of IE 1/2, UL99, UL98, and glycoprotein B. The top panel explores immediate-early, the middle panel explores early, and the bottom panel explores late protein expression. ‘U’ denotes uninfected cells, ‘I+I’ denotes infected cells with the inhibitor atanyl blue PRL at a concentration of 27.8 μ M, and ‘I’ denotes infected cells with no inhibitor. On the left the dual color standard marker is shown and the right has the respective protein size for the marker.

3. Conclusion

GFP expression is thought to occur early in the viral replication cycle. The results in figure 13 suggest that atanyl blue PRL significantly reduced GFP expression suggesting that atanyl blue PRL effects early gene expression. The spread assay (figure 15) further suggests that atanyl blue PRL reduces the amount of viral spread with time. In addition the infectivity assay (figure 17) indicates that atanyl blue PRL lowers the infectivity of the virus, and causes infected cells to become abortive and unable to produce progeny virus that can infect other cells and become a plaque. These results are consistent with the time of addition studies (figure 18) indicating that atanyl blue PRL is only effective in the first 24 hours, is most potent if added in the first 12 hours and is not very effective if added after 12 hours. Moreover, it does not appear to interact with the virus to impair attachment and entry as pretreatment had no affect. Western immunoblot analysis of IE 1/2, UL99, UL98, and glycoprotein B were also consistent with these results. Immediate-early expression was essentially eliminated in days 1 and 2 and early and late gene expression was significantly reduced. The results of GFP expression, time of addition, and western immunoblot analysis all suggest that atanyl blue PRL is an early acting compound with a profound affect on immediate-early gene expression. The effects on early and late gene expression are likely indirect results of reduced IE1/2 levels, as interference with immediate-early expression is known to have these downstream effects.

Deborah Parris, our collaborator at Ohio State University, recently demonstrated that atanyl blue PRL inhibits the nuclease activity of UL98 with an IC₅₀ of 5.7 (figure 21). Consistent with this result, docking of atanyl blue PRL into the UL98 alkaline nuclease homology model (courtesy of our collaborators Hardik Parikh and Glenn

Kellogg) indicates that atanyl blue PRL is a good fit in the UL98 alkaline nuclease active site. These results further suggests that atanyl blue PRL may express anti-CMV activity by inhibiting the UL98 alkaline nuclease.

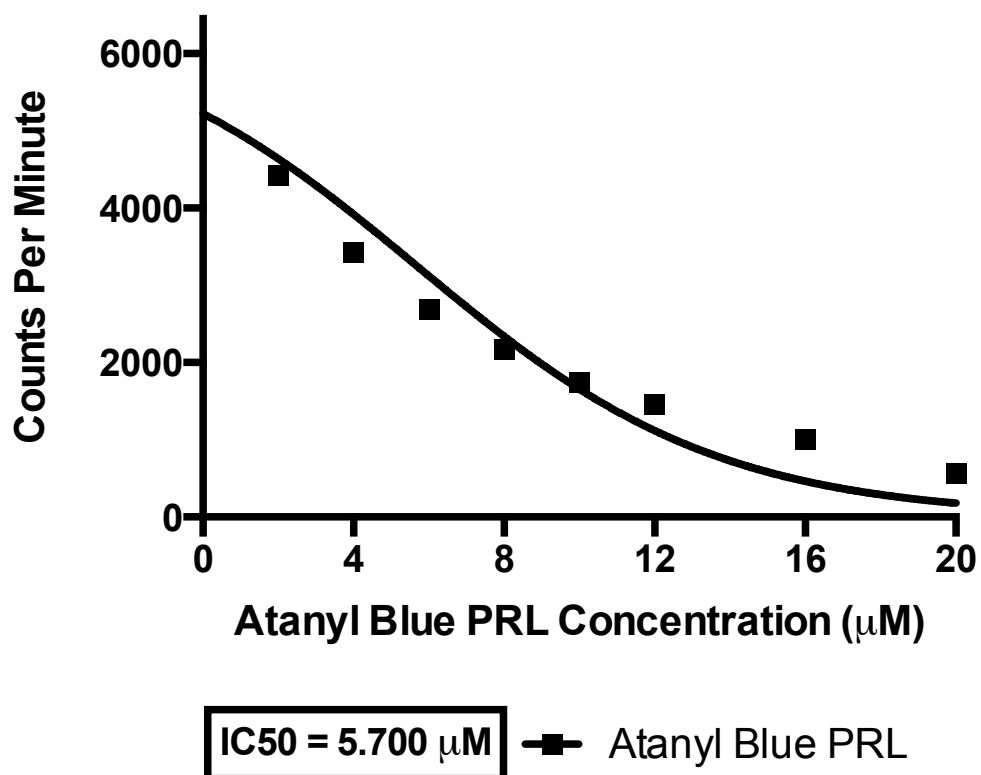


Figure 21: Inhibition of UL98 alkaline nuclease activity by atanyl blue PRL. UL98 was expressed in *E. Coli* and purified. Exonuclease activity of purified UL98 was measured using a ^{14}C -release assay (50). Unpublished data courtesy of Deborah Parris.

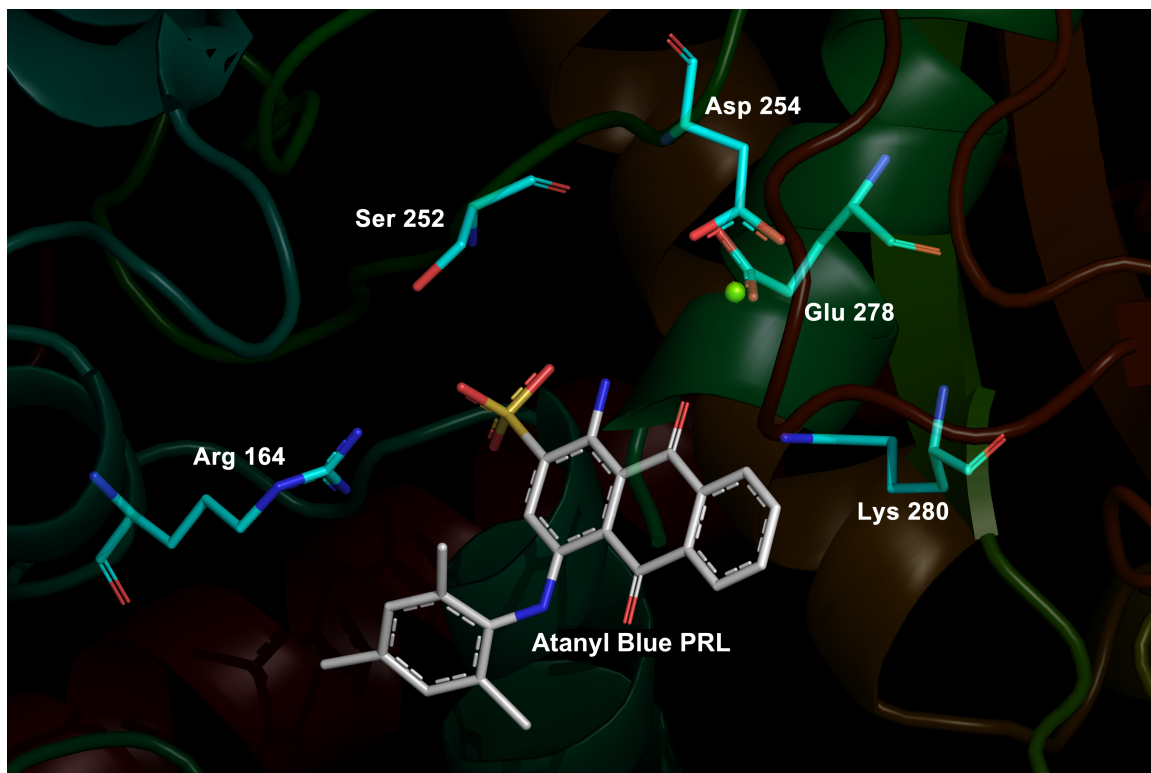


Figure 22: Docking of Atanyl Blue PRL into the UL98 homology model. Figure courtesy of Hardik Parikh and Glenn Kellogg.

IV. DISCUSSION

Cytomegalovirus, or CMV, belongs to the *Herpesviridae* family of viruses (1). The *Herpesviridae* family of viruses is a large family of double stranded DNA (120 to 235 kb) viruses. The genome is encased in an icosahedral capsid and an outer protein matrix layer known as tegument (9). The CMV genome is expressed temporally in a regulated cascade leading to synthesis of three categories of viral proteins: Immediate-early, early, and late. Immediate-early genes are the first viral genes to be expressed after a cell is infected and do not require the expression of other viral genes or protein expression. Immediate-early genes regulate the expression of early and late genes and the expression of early genes is dependent on the presence of immediate-early proteins. Transcription of immediate-early genes can occur in the absence of *de novo* protein synthesis and produces two regulatory proteins, IE1 and IE2, which play key roles in initiating and maintaining CMV gene regulation pathways to ‘jump-start’ the infection.

CMV is a relatively common infection with seroprevalence of 60% or more in persons greater than 50 years of age. It is estimated that between 45 to 100% of women of reproductive age are seropositive for CMV. The portion of women who are seronegative for CMV are at risk of primary CMV infection during pregnancy and bear a risk of giving birth to babies who will suffer from CMV-related disabilities. Most people infected with CMV are asymptomatic; however after primary infection some may show mild fever or mononucleosis-like symptoms and the host will remain infected with CMV in the latent phase for life. For immunocompromised and immunosuppressed patients, CMV is a significant cause of mortality and morbidity and it is also an important cause of birth defects in newborns if acquired in utero. (39). CMV is a major cause of mortality and morbidity in patients undergoing stem cell and solid organ transplantation (40). CMV is the most frequent opportunistic pathogen in patients infected

with HIV. Upwards of 45% of patients with AIDS are afflicted with CMV disease at some point during the course of their AIDS disease. In the United States, 40,000 children or 1% of all live births are born with an active congenital CMV infection. However, only 8,000 or 20% of those births are symptomatic. CMV infection is the most common congenital infection and a major cause of morbidity and mortality among infants (7). Infection can occur in utero, perinatally, or after birth due to contact with secretions such as breast milk and saliva of a seropositive mother (18).

Currently, there are inadequate therapies to combat CMV. With the exception of fomivirsen, all approved drugs target the viral DNA polymerase, so there is a likelihood of mutations in the DNA polymerase rendering those drugs ineffective. The drugs are also associated with significant renal and bone marrow toxicities (13). Furthermore, due to their potential toxicities, none are approved for use during pregnancy. This led to our interest in investigating novel antiviral targets. One such target, UL98, is attractive because although *UL98* null mutants are viable, viral replication in the absence of UL98 is reduced by up to 1,000-fold, indicting *UL98* is an important though not essential CMV gene (50). Relevant to our interest in CMV UL98 as a potential antiviral target, it was previously described that emodin, an anthraquinone, specifically inhibits the HSV-1 alkaline nuclease, UL12, and that emodin and several related anthraquinones also inhibit replication of CMV. Due to the need for CMV antivirals with alternative targets of action and lower toxicity, investigation of UL98 may provide a novel target for the development of new CMV antivirals. Further, anthraquinone or anthraquinone derivatives represent a novel class of anti-herpesvirals. If confirmed to act through inhibition of viral alkaline nucleases, they could serve as lead structures for structure-based drug discovery.

Our collaborators were able to use the amino acid sequence of CMV UL98 alkaline nuclease and the crystal structure KSHV-SOX protein as a template structure to model and identify the active sites of CMV UL98 (50). In order to find potential CMV UL98 inhibitors, this homology model was used to perform a virtual screening of the National Cancer Institute Open database of approximately 250,000 compounds. Those potential inhibitors were then docked, *in silico*, to the CMV UL98 model, and the 15 compounds deemed most likely to inhibit UL98 activity were selected (figure 7). Surprisingly, based on the results of the luciferase-based assay for anti-CMV activity, none of these compounds showed any substantial antiviral activity (figure 8). This could be due to inaccuracies in the homology model. A model based on the crystal structure of CMV UL98 instead of KSHV-SOX would be preferable. It is also possible that the virtual screening algorithm that was used may have missed potential inhibitors with good activity against UL98. Much larger databases of compound structures are available and they should be screened in order to find additional potential UL98 inhibitors. Finally, it is possible that some of the selected compounds are able to inhibit UL98 enzymatic activity but were unable cross the cell membrane and for this reason exhibited no anti-CMV activity in our assay. Our collaborator, Dr. Parris, plans to address this question by determining the ability of these compounds to inhibit the nuclease activity of UL98 using *in vitro*, cell-free assays.

Given the evidence that emodin inhibits HSV-1 and HSV-2 replication and interferes with the nuclease activity of HSV-1 UL12, we investigated emodin and other anthraquinones that had been previously reported to have anti-CMV activity. Emodin showed anti-CMV activity with an IC_{50} of 4.9 μM and cytotoxicity with a TD_{50} of 9.2 μM . These results are consistent with the those of Barnard *et al.* (1992), who reported an IC_{50} of 4.1 μM and TD_{50} of 9.6 μM . This rendered the antiviral effect almost indistinguishable from cytotoxicity and suggests that emodin

may act non-specifically. This was, however, inconsistent with the findings of Xiong *et al.* (2011), who observed anti-HSV activity of emodin with minimal toxicity. There is a possibility that this may be due the fact that emodin was extracted from different plants. The emodin used in our study was extracted from frangula bark, whereas Xiong *et al.* used emodin extracted from *Rheum tanguticum*, Chinese rhubarb. It is also possible that our emodin contained impurities that increased its toxicity. To address this concern a sample of the emodin used in our studies was evaluated by Ultra Performance Liquid Chromatography (UPLC) and found to be >99% pure. While a small amount of highly toxic contaminant cannot be ruled out, it appears from this result that the emodin used in our studies was not poor quality.

Acid blue 40, alizarin violet R, and atanyl blue PRL showed anti-CMV activity with IC_{50} s of 266 μ M, 238 μ M, and 6.3 μ M, respectively, and cytotoxicities with TD_{50} s of 231 μ M, 342 μ M, and 216 μ M, respectively. For acid blue 40 and alizarin violet R, any antiviral activity shown by these compounds is likely due to non-specific cytotoxicity; hence, these compounds did not exhibit any compelling antiviral activity. These results for acid blue 40 and alizarin violet R are inconsistent with those of Barnard *et al.* (1995), who reported CMV-inhibiting IC_{50} s of 10 μ M for both acid blue 40 and alizarin violet R. It is possible that these inconsistencies relate to poor solubility of these compounds. Barnard *et al.* used 0.001% tween-80 with 0.25% ethanol, which may have allowed for better solubility of the compounds. Additionally, it is possible that the DMSO used to prepare stock solutions for our studies may have interacted with these two compounds and rendered them ineffective. The cytotoxicities for these compounds, however, are relatively consistent with those of Barnard *et al.* (1995), who reported a TD_{50} of 380 μ M for acid blue 40 and a TD_{50} of 300 μ M for alizarin violet R. Further, our results for atanyl blue PRL are

consistent with the results of by Barnard *et al.* (1995), who reported an IC₅₀ of 7.0 μ M and TD₅₀ of 275 μ M. Due to these results, atanyl blue PRL was selected for further study.

Atanyl blue PRL significantly reduced GFP expression (figure 13), suggesting that atanyl blue PRL inhibits early gene expression. The spread assay (figure 15) further suggests that atanyl blue PRL reduces the amount of viral spread with time. In addition, the infectivity assay (figure 17) indicates that atanyl blue PRL lowers the infectivity of the virus and causes infected cells to become abortively infected. These results are also consistent with the time of addition studies that indicate that atanyl blue PRL is only effective when added early and virtually ineffective if added after 24 hours post infection. Atanyl blue PRL does not appear to modify the infectivity of free virus as pretreatment of free virus with the compound had no inhibitory effect. It is also unlikely that atanyl blue PRL interferes with viral attachment or entry since it was still highly inhibitory when added at 6 hours post infection, a time when entry has been completed. Further, western immunoblot analysis (figure 20) indicates that IE expression is largely eliminated by atanyl blue PRL and early and late expression is significantly reduced, most likely due to downstream affects of reduced IE protein levels. These findings are consistent with the previous results indicating that atanyl blue PRL is an early acting compound with an extensive affect on immediate-early gene expression.

Atanyl blue PRL was also found to inhibit UL98 alkaline nuclease exonuclease activity with an IC₅₀ of 5.7 μ M (figure 21), and atanyl blue PRL does appear to have a good fit into the homology model of CMV UL98 (figure 22). Hsiang and Ho (2008) found that the anthraquinone emodin inhibits HSV-1 alkaline nuclease UL12 and reduces viral yields, although it remains to be demonstrated that the mechanism of action of emodin's anti-HSV activity involves inhibition of UL12. Our studies show results that are very consistent with that of Hsiang and Ho. We found

that the related anthraquinone atanyl blue PRL inhibits the CMV alkaline nuclease, UL98, and also has anti-CMV activity. Since alkaline nucleases are highly conserved throughout the Herpesvirus family, this result was hypothesized and expected.

Given that atanyl blue PRL inhibits CMV replication and also inhibits the CMV alkaline nuclease, UL98, it is possible that its mechanism of CMV inhibition involves inhibition of UL98. However, the exact function of UL98 and when it is expressed are not fully known. Our current understanding of herpesvirus alkaline nucleases suggests that they are somehow involved in repair of viral replicative intermediate DNA prior to DNA packaging – events that occur relatively late in the replication cycle. If atanyl blue PRL inhibits CMV replication through inhibition of UL98, then our characterization of the impact of atanyl blue PRL on the CMV replication cycle suggests that UL98 may be important very early after infection. How UL98 might be important for IE gene expression is a matter of speculation. It is possible that UL98 is present in the virion particle, or is expressed *de novo* very early after infection, and has some function that promotes IE gene expression. For example, its presence in the virion might promote release of viral DNA from the capsid, circularization of viral DNA after release into the nucleus, or somehow impact DNA translocation to nuclear sites of active transcription (perhaps by degrading host DNA?). In addition, while UL98 clearly has DNase activity, it has not been evaluated for RNase activity. The alkaline nucleases of Epstein-Barr virus and KSHV have been shown to have both DNase and RNase activities and have been proposed to be analogous to HSV-1 virion host shut-off protein, a virion-associated nonspecific RNase that shuts off host protein synthesis by rapidly degrading cellular mRNAs immediately after virus entry (30, 31, 49). It is certainly conceivable that the absence of such an activity might reduce the efficiency with which viral immediate early proteins are expressed. The added corollary to the assumption

that the anti-CMV activity of atanyl blue PRL is linked to inhibition of UL98 is that UL98 activity is therefore not important for the processes of DNA synthesis or DNA packaging that occur at later times of infection (*e.g.*, atanyl blue PRL had no impact on CMV replication when added 24 hours after infection, a time well before DNA replication and packaging begins).

Alternatively, it is possible that atanyl blue PRL could inhibit UL98 and also have some other unrelated inhibitory activity that results in a to block immediate-early gene expression. Thus, inhibition of UL98 could just be coincidental and atanyl blue PRL uses some other as yet undetermined mechanism for antiviral activity. One trivial explanation is that atanyl blue PRL, due to its relatively planar anthraquinone structure, may nonspecifically impair transcription by interchelation into DNA. This explanation would seem highly unlikely given that addition of atanyl blue PRL at 24 hours post infection had no impact on viral replication. At this time early genes are just beginning to be expressed and late genes are not yet activated. Moreover, an interchelator would also be expected to inhibit viral DNA synthesis, which is only just starting at 24 hours post infection. Thus, non-specific inhibition of RNA transcription or DNA synthesis at 24 hours post infection or later would be expected to cause major reductions in virus yield.

Future studies into atanyl blue PRL will involve creating atanyl blue PRL-resistant viruses, mapping the mutations responsible for resistance, and determining if those mutations lie in *UL98*. If mutations that confer resistance are identified in *UL98* then it is quite conclusive that atanyl blue PRL is specifically affecting CMV replication through inhibition of *UL98*. Also, *UL98* null mutants could be used and examined for resistance to atanyl blue PRL. If there is no change in the growth of the mutants, this it would suggest that *UL98* is the only target. However, if there is further attenuation of viral growth, then there must be another mechanism that atanyl blue PRL uses to target some other important viral function.

Formal confirmation that atanyl blue PRL acts by inhibiting UL98 is important for two reasons. First, this would establish that UL98 activity is critically important only at very early times of infection. As discussed above, this would be paradigm-shifting for our understanding of how alkaline nucleases function, at least in the context of CMV replication. Second, atanyl blue PRL could serve as a lead structure for further structure-function studies to derive more active inhibitors that would presumably have greater antiviral potency. Finally, if it can be demonstrated that atanyl blue PRL has anti-CMV activity against animal CMVs such as murine CMV, guinea pig CMV, or rhesus CMV, then animal studies of antiviral efficacy could be considered.

LITERATURE CITED

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1. Yamanishi K, *et al.* (2007). Human herpesvirus: Biology, therapy, and immunoprophylaxis. *Cambridge University Press*.
2. Cannon MJ, *et al.* (2010). Review of cytomegalovirus seroprevalence and demographic characteristics associated with infection. *Review of Medical Virology*, 20(4), 202-213.
3. Ljungman P, *et al.* (2002). Definition of cytomegalovirus infection and disease in transplant recipients. *Clinical Infectious Disease*, 34(8), 1094-1097.
4. Van Der Bij W, *et al.* (2001). Management of cytomegalovirus infection and disease after solid-organ transplantation. *Clinical Infectious Disease*, 33(7), 532-537.
5. Jab DA (1996). Treatment of cytomegalovirus retinitis in patients with AIDS. *Annals of Internal Medicine*, 125, 144-145.
6. Sugar EA, *et al.* (2012). Studies of the ocular complications of AIDS research group: Incidence of cytomegalovirus retinitis in the era of highly active antiretroviral therapy. *American Journal of Ophthalmology*, 153(6), 1016-1024.
7. Michaels MG (2007). Treatment of congenital cytomegalovirus: Where are we now? *Expert Review of Anti-infective Therapy*, 5, 441-448.
8. Adler SP (2005). Congenital cytomegalovirus screening. *The Pediatric Infectious Disease Journal*, 24(12), 1105-1106.
9. Mettenleiter TC, *et al.* (2008). Molecular biology of animal herpesviruses. *Animal Viruses: Molecular Biology*. Caister Academic Press.
10. Yang S, *et al.* (2005). Using DNA microarray to study human cytomegalovirus gene expression. *Journal of Virological Methods*, 131(2), 202-208.
11. Landolfo S, *et al.* (2003). The human cytomegalovirus. *Pharmacology and Therapeutics*, 98, 269-297.
12. Marschall M, *et al.* (2009). Molecular targets for antiviral therapy of cytomegalovirus infections. *Future Microbiology*, 4(6), 731-742.
13. Biron KK (2006). Antiviral drugs for cytomegalovirus disease. *Antiviral Research*, 71, 154-160.
14. Sullivan V, *et al.* (1993). A point mutation in the human cytomegalovirus DNA polymerase gene confers resistance to Ganciclovir and phosphonylmethoxyalkyl derivatives. *Antimicrobial Agents and Chemotherapy*, 37(1), 19-25.

15. Sugawara M, *et al.* (2000). Transport of valganciclovir, a ganciclovir prodrug, via peptide transporters PEPT1 and PEPT2. *Journal of Pharmaceutical Sciences*, 89(6), 781-790.
16. Gilbert C, *et al.* (2005). Human cytomegalovirus resistance to antiviral drugs. *Antimicrobial Agents and Chemotherapy*, 49(3), 873-883.
17. Sokol DM (1996). Foscarnet. *Seminars in Pediatric Infectious Diseases*, 7(2), 145-147.
18. Griffiths PD, *et al.* (1984). A prospective study of primary cytomegalovirus infection during pregnancy: Final report. *British Journal of Obstetrics and Gynaecology*, 91, 307-315.
19. O'Brien JJ, *et al.* (1989). Aciclovir: An updated review of its antiviral activity, pharmacokinetic properties and therapeutic efficacy. *Drugs*, 37(3), 233-309.
20. Roush W (1997). Antisense aims for a renaissance. *Science*, 276(5316), 1192-1193.
21. Mettenleiter TC (2004). Budding events in herpesvirus morphogenesis. *Virus Research*, 106(2), 167-180.
22. Hsiang CY, *et al.* (2008). Emodin is a novel alkaline nuclease inhibitor that suppresses herpes simplex virus type 1 yields in cell cultures. *British Journal of Pharmacology*, 155(2), 227-235.
23. Barnard DL, *et al.* (1995). Anti-human cytomegalovirus activity and toxicity of sulfonated anthraquinones and anthraquinone derivatives. *Antiviral Research*, 28(4), 317-329.
24. Barnard DL, *et al.* (1992). Evaluation of the antiviral activity of anthraquinones, anthrones, and anthraquinone derivatives against human cytomegalovirus. *Antiviral Research*, 1, 63-77.
25. Bien HS, *et al.* (2005). Anthraquinone dyes and intermediates. *Ullmann's Encyclopedia of Industrial Chemistry*.
26. Goor G, *et al.* (2007). Hydrogen Peroxide. *Ullmann's Encyclopedia of Industrial Chemistry*.
27. Müller-Lissner SA (1993). Adverse effects of laxatives: Fact and fiction. *Pharmacology*, 47(1), 138-145.
28. Sydiskis RJ, *et al.* (1991). Inactivation of enveloped viruses by anthraquinones extracted from plants. *Antimicrobial Agents and Chemotherapy*, 35(12), 2463-2466.
29. Xiong HR, *et al.* (2011). The effect of emodin, an anthraquinone derivative extracted from the roots of *Rheum tanguticum*, against herpes simplex virus *in vitro* and *in vivo*. *Journal of Ethnopharmacology*, 133(2), 718-723.
30. Glaunsinger B, *et al.* (2004). Lytic KSHV infection inhibits host gene expression by accelerating global mRNA turnover. *Molecular Cell*, 13(5), 713-723.

31. Rowe M, *et al.* (2007). Host shutoff during productive Epstein-Barr virus infection is mediated by BGLF5 and may contribute to immune evasion. *Proceedings of the National Academy of Sciences of the United States of America*, 104(9), 3366-3371.
32. Anderson DO, *et al.* (1991). *In vitro* virucidal activity of selected anthraquinones and anthraquinone derivatives. *Antiviral Research*, 16(2), 185-196.
33. Semple SJ, *et al.* (2001). *In vitro* antiviral activity of the anthraquinone chrysophanic acid against poliovirus. *Antiviral Research*, 49(3), 169-178.
34. Roxas M, (2006). Herpes zoster and postherpetic neuralgia: diagnosis and therapeutic considerations. *Alternative Medicine Review*, 11(2), 102-113.
35. Shors, T. (2011). *Understanding Viruses. (2 ed.). Burlington, MA: Jones & Bartlett Learning.*
36. Hahn G, *et al.* (2009). Human cytomegalovirus UL131-128 genes are indispensable for virus growth in endothelial cells and virus transfer to leukocytes. *Journal of Virology*, 78(18), 10023-10033.
37. Cohen S, *et al.* (2011). How viruses access the nucleus. *Biochimica et Biophysica Acta*, 1813(9), 1634-1645.
38. Mettenleiter, TC (2002). Herpesvirus assembly and egress. *Journal of Virology*, 76(4), 1537-1547.
39. Ho, M. (1991). *Cytomegalovirus: Biology and Infection. Pittsburg, PA: Springer publishing.*
40. Ghandi MK, *et al.* (2004). Human cytomegalovirus: Clinical aspects, immune regulation, and emerging treatments. *The Lancet Infectious Diseases*, 4(12), 725-738.
41. Anderson D, *et al.* (2010). Introduction: Disease overview and risk factors. *Pharmacy and Therapeutics*, 35(3), 2-4.
42. Fowler KB, *et al.* (1997). Progressive and fluctuating sensorineural hearing loss in children with asymptomatic congenital cytomegalovirus infection. *Journal of Pediatrics*, 130(4), 624-630.
43. Nelson CT, *et al.* (1997). Cytomegalovirus infection in the pregnant mother, fetus, and newborn infant. *Clinics in Perinatology*, 24(1), 151-160.
44. Fowler KB, *et al.* (1999). Newborn hearing screening: Will children with hearing loss caused by congenital cytomegalovirus infection be missed? *Journal of Pediatrics*. 135(1), 60-64.
45. Andrei G, *et al.* (2008). Novel inhibitors of human CMV. *Current Opinion in Investigational Drugs*, 9(2), 132-145.

46. Wang D, *et al.* (2004). Human cytomegalovirus encodes a highly specific RANTES decoy receptor. *Proceedings of the National Academy of Sciences of the United States of America*, 101(47), 16642-16647.
47. Wang D, *et al.* (2005). Human cytomegalovirus UL131 open reading frame is required for epithelial cell tropism. *Journal of Virology*, 79(16), 10330-10338.
48. McVoy MA, *et al.* (1999). Tetracycline-mediated regulation of gene expression within the human cytomegalovirus genome. *Virology*, 258(2), 295-303.
49. Everly DN, *et al.* (2002). mRNA degradation by the virion host shutoff (VHS) protein of herpes simplex virus: genetic and biochemical evidence that VHS is a nuclease. *Journal of Virology*, 76(17), 8560-8571.
50. Kuchta AL, *et al.* (2012). Structural modeling and mutagenesis of human cytomegalovirus alkaline nuclease UL98. *Journal of General Virology*, 93(1), 130-138.
51. Boeckh M, *et al.* (2003). Cytomegalovirus in hematopoietic stem cell transplant recipients: Current status, known challenges, and future strategies. *Biology of Blood and Marrow Transplantation: Journal of the American Society for Blood and Marrow Transplantation*, 9(9), 543-558.

VITA

Zohaib Alam
alamz2@vcu.edu

Zohaib Alam was born on August 16, 1987, in Gothenburg, Sweden and is an American and European Union citizen. He graduated from Hermitage High School, Richmond, Virginia in 2004. He graduated cum laude and received his Bachelor of Science in Biology with a minor in Chemistry and Bachelor of Arts in Religious Studies in 2009 from Virginia Commonwealth University, Richmond, Virginia.